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Calcium and Leucine Modulation of Airway Inflammation

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To the Graduate Council:

I am submitting herewith a dissertation written by Patricia Louise Brown entitled "Calcium and Leucine Modulation of Airway Inflammation." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

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Abstract

Over the past several decades the prevalence of obesity and asthma have increased in a parallel fashion. Recent studies reported a positive relationship between the two disorders that may in fact be causal. Although the link between obesity and asthma has become widely recognized, the underlying pathophysiological connection is not elucidated. Increased markers of inflammatory and oxidative stress are present in obesity and asthma suggesting the link is immunological. The systemic inflammation observed in obesity may potentially initiate adverse affects in the airways. Previous studies have shown that consumption of dairy foods (rich in calcium and leucine) suppress 1,25-dihydroxycholecalciferol (calcitriol) resulting in decreased inflammatory stress associated with excess adiposity. Additionally, adipocyte leucine treatment was reported to decrease pro-inflammatory TNFα and increase anti-inflammatory adiponectin cytokines, which have been implicated in asthmatic disease. Consequently, we sought to determine if correcting the imbalance of adipocyte inflammatory cytokine secretion via calcium and leucine treatment would have a functional effect on airway inflammation. We demonstrated that conditioned medium collected adipocytes (ACM) treated with leucine for 48hrs significantly reduced monocyte-airway smooth muscle adhesion, lung endothelial cell ICAM-1 adhesion molecule expression, and polymorphonuclear (PMN) cell CD11b expression *in vitro* compared to control, while calcitriol exerted the opposite effects. Furthermore, these findings were extended to an established murine model of asthma. Female BALB/c mice were sensitized and challenged with chicken egg albumin (OVA) to induce airway inflammation. Animals were fed a high fat diet with no supplementation, high calcium (1.2%), leucine (200% normal levels), or a diet with a

combination of calcium (1.2%) and leucine (200% normal levels). We found that the combined high calcium and leucine supplemented high fat diet animals had significantly less eosinophils in collected bronchoalveolar lavage fluid (BALF) compared to control diet mice. These data suggest that calcium and leucine may have potential therapeutic affects on obesity associated airway inflammation.

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List of Abbreviations and Symbols

Calcitriol	1,25-Dihydroxycholecalciferol
ACM	Adipocyte Conditioned Medium
ICAM-1	Intracellular adhesion molecule
VCAM-1	Vascular adhesion molecule
M:E	monocyte to endothelial cell ratio
ROS	reactive oxygen species
TNF-α	tumor necrosis factor alpha
IL-6	interleukin 6
UCP1	uncoupling protein 1
UCP2	uncoupling protein 2
MCP-1	monocyte chemotactic protein 1
MIF	macrophage migration inhibitory factor
CD11b	cluster of differentiation molecule 11b
CD54	cluster of differentiation molecule 54
IL-8	interleukin 8
BALF	Bronchial alveolar lavage fluid
BALT	Bronchus Associated Lymphoid Tissue (BALT)
HypEpi	Lung Epithelial Hyperplasia
VEGF	vascular endothelial growth factor
DiI	1,1′-Dioctadecyl-3, 3, 3′, 3′- tetramethylindocarbocyanine perchlorate
PBS	phosphate buffered saline
HAT	Hypoxanthine - Aminopterin – Thymidine
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
SIRT1	sirtuin 1
SEM	standard error mean

Part One

Introduction

Introduction

Asthma prevalence has increased in industrialized nations over the past several decades (1). The increase in asthma diagnosis has been observed in all ages, genders, and racial groups (1). Over 300 million people suffer from asthma globally with over 25 million people living with asthma in the United States alone (2-5). The obesity epidemic and asthma have grown in a parallel fashion and there is an increasing body of evidence to suggest a positive relationship between the two disorders (6,7). Prospective studies have shown that increasing BMI correlates with an increased risk of asthma development while weight loss improves the course of the illness (8,9). Obesity is acknowledged as a predisposing factor for asthma development. However, the underlying pathophysiological connection remains elusive. As both asthma and obesity share many of the same inflammatory cytokines and oxidative stress markers a likely connection may be immunological (10-12). The chronic low-grade systemic inflammation and oxidative stress may work synergistically to adversely modify the airways. The balance of inflammatory and anti-inflammatory cytokines secreted by adipose tissue is altered by oxidative stress (13). The increase in reactive oxygen species (ROS) suppresses adipose tissue expression of adiponectin and increases expression of inflammatory factors, including TNFα, while suppression of ROS exerts the opposite effect (13). Mitochondrial uncoupling status and cytosolic Ca^{2+} signaling, both of which are regulated by calcitriol (vitamin D_3) modulate adipocyte ROS production (14-16). Calcitriol suppresses the expression of adipocyte uncoupling protein 2 (UCP2), resulting in greater mitochondrial membrane potential, and stimulation of cytosolic Ca^{2+} signaling, both of which lead to increased ROS production (14-17). We have recently shown physiological concentrations

of calcitriol (100pm-10nM) similar to levels observed in calcium deficiency stimulated the expression and secretion of inflammatory cytokines, including TNFα, while inhibiting adiponectin expression and secretion (14-17). These concentrations of calcitriol stimulate ROS production in both murine and human adipocytes (14-17). Further, we have shown calcitriol to exert comparable effects in macrophages and to stimulate macrophage-adipocyte cross talk and inflammatory response in co-culture (17).

Consequently, we evaluated the ability of dietary calcium-induced suppression of circulating calcitriol to attenuate oxidative and inflammatory stress and found significant, substantial suppression of both adipose tissue and systemic oxidative and inflammatory stress in a mouse model (14, 18). These findings were extended to a randomized, blinded cross over clinical trial in overweight and obese subjects where it was found that suppressing circulating calcitriol with a calcium-rich diet suppresses systemic oxidative stress by ~20%, reducing TNF-α and increased adiponectin by 20% ($p<0.002$)(18). Recent studies support a role for leucine in attenuating oxidative and inflammatory stress has been supported (19,20). We have found 0.5 mM leucine (comparable to levels achieved following a leucine-rich meal) to stimulate mitochondrial biogenesis in both adipocytes and muscle cells, with an associated increase in oxygen consumption and fatty acid oxidation in both cell types (20). This increase in energy utilization reduces the oxidative and inflammatory stress that otherwise results from nutrient overload and obesity.

The purpose of this work was to determine if leucine and calcium treatment have a functional effect on the obesity associated airway inflammatory process using both *in vitro* and *in vivo* models.

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Part Two

Literature Review

2.1 Asthma

2.1.1 Overview

Asthma is an inflammatory disease of the airways affecting more than 300 million people worldwide[1] with over 25 million living in the United States in 2010[2-4]. Urbanized nations such as the US have reported increases in asthma prevalence among all racial groups, ages, and genders over the past thirty years [5]. As the leading childhood chronic illness, asthma affects more than ten million American adolescents and children[6]. By the year 2025, the number of childhood asthmatics in the US is expected to exceed 100 million[7]. Although modern treatment has helped lower mortality[8],over 250,000 people die annually from asthma related symptoms[9]. Asthma can affect people of any age. The term 'phenotype' has been initially used to describe the heterogeneity in asthmatic manifestations[10]. Asthmatic phenotypes/endotypes are characterized based on criteria such as: presence of allergy, severity, pathology (eosinophilic, noneosinophilic) and age of onset among other factors[11-17]. Endotype is a relatively new concept in asthma classification[18]. The term was introduced to specially describe subphenotypes in asthma that include distinctive or unique pathological or functional mechanism such as aspirin-sensitive asthma, early onset allergic asthma, neutrophilic and obesity- related, Th2 associated, severe late onset eosinophilic, non Th2 associated and bronchopulmonary mycosis[13]. Collection of adequate laboratory and clinical data on each asthmatic patient is needed before an appropriate definition of specific phenotypes can be determined[18]. Therefore, characterizations of all the distinct asthmatic phenotypes and pathologies have yet to be fully elucidated.

2.1.2 Disease Description

Many different cell types participate in the pathology of asthma mast cells and eosinophils are critical in the airway inflammatory process while leukotrienes, cytokines, histamine mediators and T lymphocytes are also involved[10]. Pathologies such as airway inflammation as well as airway hyper-responsiveness (AHR) associated with reversible airflow obstruction are traditional attributes of asthmatic disease[19]. Classic clinical symptoms of asthma patients are recurrent episodes of wheeze, shortness of breath, and coughing due to abnormalities in the airways[2]. Several other symptoms such as bronchial hyper-responsiveness and airway obstruction typically associated with asthma are also pathologically linked to other disorders such as obstructive sleep apnea, obesity, stressors, reflux or atopy[20]. Asthma attacks (acute exacerbations) pose the biggest threat to the asthmatic patient. Exacerbations are episodes of airway obstruction (chest tightness, shortness of breath, coughing, and wheezing) that may result in loss of lung function or death[21]. Asthmatics experience these recurrent episodes of airflow obstruction, airway hyper-responsiveness and inflammation in response to irritants, allergens, cold air, or exercise exposure[22]. Symptom control is crucial to improving the severity and frequency of life threatening acute exacerbations. Defining the underlying pathologies of distinct asthmatic phenotypes is the key to providing proper individualized treatments[22].

2.1.3 Pathology

The underlying pathophysiological mechanism of asthmatic disease involves a dysfunctional multisystem response including airway epithelium, airway smooth muscle, and circulatory system[22]. Histological changes involve epithelial damage, thickening of

the basement membrane, mucus production and airway smooth muscle (ASM) thickening.[22,23] The frontline defense for healthy airways against allergens or stimuli in the environment is the airway epithelium[24]. Asthmatics however, suffer from incomplete formation of tight junctions in the airway epithelium that are defective in preventing allergen infiltration into the airway tissue[24]. Damage to epithelium and composing epithelial cells is a result of chronic airway inflammation[20]. This airway inflammation is a result of leukocyte infiltration and subsequent cytokine secretion. Eosinophils are one of the first major cell types recruited to sites eliciting an inflammatory response[25-27]. Asthma development and progression are affected by eosinophil secretion of lipid mediators, reactive oxygen species (ROS), cytokines, and toxic granule proteins[28]. Eosinophils play an active role in asthmatic airway remodeling through several mechanisms. Interactions with epithelial and mesenchymal cells, release of cationic proteins, cytokines, and eosinophil-derived TGF-β are all contributors to eosinophil influenced airway remodeling[29-31]. Necrotic airway epithelial cell cellular debris, mucin produced by goblet cells, eosinophils, lymphocytes, neutrophils, and plasma protein exudate make up the composition of mucous[32-34]. Over the past several years there has been an emerging interest in asthma airway remodeling mechanisms[35]. Remodeling and inflammatory processes work interdependently to induce structural changes in the asthmatic airway[36]. Airway remodeling has been suggested to be a consequence of injured airway epithelial cells that work as an incessant stimulus for the process[35]. Reactive oxygen species (ROS) generated from resident inflammatory cells in the lung are key contributors to asthmatic pathophysiology[37]. Diminished β-adrenergic receptor response, airway hyperresponsiveness, elevated vascular permeability, mucus hypersecretion, secretion of

neuropeptides, and release of chemoattractants can all result from the production of reactive oxygen species [37-42]. Numerous enzymatic and non-enzymatic antioxidants work to protect the lungs and blood from ROS generated from mitochondria as a consequence of regular cellular metabolism[43]. Obesity increases systemic oxidative stress which alters adipokine secretion by decreasing anti-inflammatory adiponectin while increasing inflammatory cytokine secretion[44-46]. Oxidants induce the inflammatory process while antioxidants reduce oxidative stress and ameliorate adverse adipokine secretion[44-46]. It remains unclear which of these aspects of pathogenesis is the initial stimulus for disease manifestation. Traditionally, the classic asthmatic phenotype presents high IgE levels accompanying eosinophilia in the airways[20].

2.1.4 Airway Inflammation

Asthmatics suffer from chronic airway inflammation[20]. Inflammatory cells, such as leukocytes, are responsible for secretion of potent chemical mediators that initiate inflammation in the airways[20]. Classically, T helper type 2 (Th2) lymphocytes were thought to be solely responsible for asthma initation[20]. First, antigen presenting cells such as dendritic cells (DCs) would take up allergens and present them to naïve T helper cell lymphocytes[20]. Allergens such as pollens, house dust mites, animal dander, fungi are common inhaled stimulants that can be responsible for asthma sensitization[20]. As a consequence of sensitization allergen specific Th2 cell production is initiated[20]. Proliferation of Th2 cells stimulated by inhaled allergens increase production and release of cytokines such as interleukin (IL)-4, IL-5, and IL-13, which contributes to asthma development[20]. Modern characterization has incorporated Th9 and Th17 lymphocytes as well in disease modulation[20]. It has been reported that airway inflammation can be

induced by Th17 produced IL-22, IL-17A, and IL-17F[20]. Additionally, IL-17A enhances the contractility of airway smooth muscle cells[20].

Histological changes involve epithelial damage, basement membrane thickening, mucus production and airway smooth muscle (ASM) thickening[22,23]. Although it is not clear which of these factors is the most important, airway smooth muscle is an essential element of acute airway narrowing[23]. The increased smooth muscle mass in different individuals may be caused by hypertrophy and/or hyperplasia[23]. However, since mammalian cells must undergo cell enlargement prior to mitosis, cell growth and increased contractile protein expression is common to both[47]. Multiple signaling pathways and growth factors are involved in regulating ASM proliferation. These include the phosphaditylinositol 3-kinase (PI3K) pathway, the extracellular single-regulated kinase (ERK) pathway and peptides such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)[23].

Airway inflammation by leukocyte infiltration into airways is one of the staple characteristics of asthmatic disease[26]. Polymorphonuclear cells (PMNs) leukocytes include neutrophils, eosinophils, and basophils[30]. PMN subtypes have diverse roles in immunological responses. Eosinophils, neutrophils, and mononuclear leukocytes such as monocytes have been implicated in airway diseases[27]. The β2-integrin CD11b is expressed continuously on multiple leukocyte sub populations including eosinophils, neutrophils, and monocytes[27]. After stimulation with a cytokine or other mediator of stress, leukocytes are activated and increase levels of CD11b integrin expression on the cell surface[27]. This increased expression is needed for proper guidance and attachment to resident cell types at the site of inflammation[27]. Upon reaching the site of inflammation

via chemotactic guidance, eosinophils, neutrophils, and monocytes begin rolling on P and E selectins expressed on endothelial cells[27]. Before infiltration is initiated the leukocyte anchors via adhesion molecules[27].

Intracellular adhesion molecule (ICAM-1) is present on vascular and pulmonary endothelial cells as well as smooth muscle cells[48]. Upon injury or stimulation from stress the expression of ICAM-1 surpasses normal levels and awaits arrival and attachment of leukocytes[48]. Adhesion molecules such as ICAM-1 anchors the leukocyte and guides infiltration into tissue[48]. Once leukocytes are present in the tissue, cytokine and chemokine secretion is amplified[48]. The newly present mediators of inflammation recruits more leukocytes and initiates remodeling as a consequence of ongoing inflammation[48].

2.1.5 Cytokines in Atopic Asthma

The staple cytokines in classical asthma are encoded on chromosome 5q31 in the IL-4 gene cluster[50]. Cytokines encoded on the gene cluster such as IL-4, IL-5, IL-13, IL-9 as well as granulocyte colony stimulating factor (GM-CSF) contribute to asthma is different ways[50]. IL-4 is a Th2-cell-associated cytokine that promotes increased expression of adhesion molecules, B-cell isotype switching, facilitates goblet cell metaplasia, and airway hyperresponsiveness (AHR) and stimulates eotaxin secretion[20,51].

2.1.6 Asthma Disparities

Although the prevalence of asthma has grown in urbanized nations in the past several decades among all populations[52], the largest percentage increase is observed in children[3]. Traditionally thought of as an allergic disease it seems fitting that children with allergies are more likely to develop asthma[53,54]. However, recent findings have reported non-allergy related asthma in a large population of childhood patients[53-55]. These types of

inhaled allergen sensitizations are thought to be the reason most asthma cases start in childhood[20].

Although more obvious in obese women compared to obese men[56], there is an increasing body of evidence for an association between asthma and obesity[57-59]. Several cross-sectional studies have found obesity to be a predisposing factor in both children and adults for asthma onset[60]. The underlying mechanism connecting obesity and asthma is a controversial research topic. One theory proposes the increase in inflammatory and oxidative stress observed in obesity to synergistically harm the airways[61]. Increased pro-inflammatory cytokine production from excess adipose tissue observed in obesity has been associated with asthma[61]. It has also been suggested that the adipokine-asthma association is age and sex dependent as the link is more pronounced in premenopausal women, prepubertal boys, and peripubertal girls[62,63]. Perhaps there is a hormone ratio guiding the T-cell responses in addition to excess adiposity resulting in distinguished phenotypes[64]. Increased estrogen levels as experienced in phases of sexual maturity and increased adiposity greatly influence immunological responses and may amplify obesity related pro-inflammatory cytokine secretion[64]. A combination of symptoms, the degree of bronchial obstruction, dose of inhaled corticosteroids needed for asthma control, intensity of treatment, and inflammatory markers are criteria used to define severe asthma[58]. Frequent exacerbations in severe asthma are associated with comorbidities such as obesity, gastroesophageal reflux, obstructive sleep apnea syndrome (OSAS), and psychological dysfunction[65].

2.1.7 Current Asthma Therapies

Albeit pathological descriptions of asthmatic disease give insight into the established mechanistic aspects of the disease, they are not appropriate for clinical purposes[66]. More practical in primary care setting breathlessness, coughing, wheezing, and tightening of the chest define the disease, which fluctuates over time in intensity, frequency and occurrence[66]. Often it is the alleviation of these symptoms that are used to measure treatment response[67].

There are several medications available to control asthma: inhaled glucocorticosteroids, leukotriene modifiers, long-acting inhaled β_2-agonists (LABA)s in combination with glucocorticosteriods, systemic glucocorticosteroids, theophylline, cromones, and anti-IgE. Reliever medications which include rapid acting inhaled β_2-agonists, systemic glucocorticosteroids, anti-cholinergics, theophylline, and short-acting oral β_2-agonists[67]. Most commonly, asthma is treated by inhaled corticosteroids (ICS) as well as bronchodilators to improve quality of life and alleviate the frequency of symptoms[67]. There are subsets of asthmatics that do not respond well to standardized treatments[67]. Particularly, asthmatics that are also defined as obese seem to have a treatment refractory phenotype[67]. A decreased response to inhaled corticosteroids used in asthma management has been observed in overweight or obese children[67]. Severe asthmatics who are of normal weight are significantly more likely to exhibit optimal asthma control compared to overweight subjects[68]. Further, the optimal control state was completely missing in severe asthmatics that were obese[68]. From these observations it appears that severe asthmatic patients with good nutritional status seem to correlate with better "controlled" pulmonary disease management[68]. These findings agree with previous

studies that have shown that normal weight asthmatics have better disease control than obese patients[69]. The recently observed neutrophil rich airway inflammation of obese asthmatics may explain the treatment refractory subtype associated with excess adiposity[70,71].

2.2 Adipose Tissue

2.2.1 Adipocytes

Adipose tissue is found in two major types throughout the human body, the first and most abundant in adults is white adipose tissue (WAT) the second is more prominent in infants and is termed brown adipose tissue (BAT)[72]. White adipocytes are filled with a single large lipid droplet and are spherical[72]. Adipocytes interact with the heart, liver, and skeletal muscle as a very important component of metabolic processes[73]. After a meal rich in fat, adipocytes will store TAG absorbed from the intestinal tract (chylomicrons) or the liver (VLDL) until needed for fuel[73]. When additional fuel is needed for physiological processes TAGs will by hydrolyzed to release free fatty acids by adipocyte lipases[73]. Adipose tissue mass depends on the number of adipocytes and volume of each cell[74]. The volume of adipocytes is determined by the removal and storage of TAGs[75]. Lipid turnover or rate of TAG removal refers to the hydrolysis of TAGs (lipolysis) to remove lipid stores from adipocytes, which is then followed by oxidation that is an irreversible process[76]. Obesity (excess adipose tissue) alters rate of lipid turnover and hence decreases the removal rate of TAGs[76].

2.2.2 Obesity

Obesity is a global epidemic with extremely expensive healthcare consequences[77,78]. Cancer, cardiovascular disease, type II diabetes, and asthma are among

many consequences of obesity[73]. Obesity is determined by a body mass index (BMI) of ≥ 30 calculated by body weight in kg divided by the height in meters squared (kg/m^2)[72]. Currently, in the United States over 30% of adult men and women are considered obese and 35% are overweight (BMI >25)[72]. A BMI below 25 is considered normal; 25-29.9 is overweight and ≥30 is characterized as obese[72]. Obesity is a consequence of consistent intake of more calories than are expended resulting in unused calories and subsequent excess adiposity[72]. The excess of dietary calories can be used in several ways. First, excess calories can be utilized by treating them as "waste" using them as fuel for thermogenesis (heat production) in mitochondria that are upcoupled[72]. Second, the excess calories can be burned by increased expenditure such as exercise[72]. Third, the unused calories can be converted to fat and stored in adipose tissue[72].

Under normal conditions adipose tissue will clear the TAGs in circulation following intake of dietary lipids[79,80]. By this process, adipose tissue prevents free fatty acids from being released into circulation[79,80]. Obese subjects with excess visceral fat experience a failure of adipose tissue to store the amount of excess free fatty acids and TAG[79,80]. The dysfunctional adipose tissue leads to extremely high levels of systemic free fatty acids and TAG[79,80].

2.2.3 Obesity and Adipose Tissue Inflammation

Elevated levels of free fatty acids as that observed in obesity have the ability to activate the innate immune response[81]. Through interaction with Toll-like receptor-4 (TLR-4) free fatty acids initiate a battery of adipose tissue inflammatory pathways[79,82]. Pathways contributing to the adipose tissue inflammatory cascades include NLRP3 inflammasome-mediated[83-85], endoplasmic reticulum (ER)-stress mediators[86], and TLR[82]

regulated pathways[79]. The increased systemic inflammation observed in obesity is largely a result of these activated inflammatory pathways[64]. As a consequence of inflammatory cascades in obesity adipose tissue becomes an active endocrine organ[87]. The term "metaflammation"or metabolic inflammation is the result of obesity induced adipose tissue endocrine function[80,81]. Chronic excessive calorie intake leads to increased lipid filling in white adipocytes resulting in cytokine release and apoptosis[73]. Upon adipocyte apoptosis there is a release of free fatty acids by breakdown of stored TAG[73]. Monocytes are recruited to adipose tissue due to increases in cytokine and chemokine secretion[73]. Macrophages undergo a transformation from the M_2 phenotype to the inflammatory M_1 phenotype associated with obesity[73]. These activated macrophages ingest adipocyte debris by phagocytosis[73]. Activated adipose tissue M_1 macrophages contribute to increased levels of inflammatory cytokines such as TNFα[73], monocyte chemoattractant protein-1 (MCP-1)[88], IL-6[89]. This inflammatory adipose tissue increases inflammatory and oxidative stress systemically as well as locally[73]. It is this inflammation that links obesity to insulin resistance and subsequent development of diabetes mellitus, cardiovascular disease, metabolic disorders, cancer, and airway disease[73]. Endoplasmic reticulum (ER) stress along with protein kinases such as c-Jun *N* terminal kinase (JNK) and IκB kinase IKK that initiate release of inflammatory cytokines from adipose tissue are induced by excess nutrients such as free fatty acids (FFA)[79]. Over production of IL-1β due to increased number of macrophages in obese adipose tissue leads to increased airway hyperresponsiveness and proliferation as well as activation of IL-17 producing cells both of which are fundamental features of asthma[66,90].

Obesity increases M1 activated macrophages as well CD4+ Th1 cells mast cells and CD8+ effector cells[85]. Decreased in the obese state are M2 macrophages Th2 cells, T reg cells, and eosinophils.

2.3 Adipokines

2.3.1 Cytokines in Adipose Tissue

Adipose tissue secretes adipocytokines or adipokines as well as other inflammatory mediators that significantly contribute to the systemic inflammation in obesity[87,91]. Once thought as just a tissue responsible for storing energy reserves adipose tissue has emerged as an active endocrine organ[73]. Adipokines or peptide hormones produced by adipocytes have the ability to work locally (autocrine and paracrine) or systemically (endocrine)[72]. These adipokines relay information to the brain and to other tissues in the body about the content of energy reserves in adipocytes[72]. The first theory postulated about adipose tissue involvement as an endocrine organ was termed the "adiposity negative-feedback" model[72]. This theory postulated that when the body weight exceeded a particular value (set point) a protective mechanism would take into effect that would increase energy expenditure and inhibit eating behavior to prevent further weight gain[72]. The model predicted that the part of the brain responsible for metabolic and motor activity was influenced by a signal originated in adipose tissue[72]. Evidence of an adipocyte derived signaling factor that matched the postulated model came with the early nineties discovery of the adipokine leptin[72]. Under normal conditions, leptin modulates eating behavior and fuel metabolism to maintain body mass and adequate fuel reserves[72]. However, any alteration in adipokine production such as that observed in obesity leads to dysregulation and subsequent chronic metabolic related diseases[72]. Adipokine over or

under production is the principal link of obesity to the low grade systemic inflammation now considered characteristic of the disease[72]. The increased inflammatory cytokines have been proposed to link obesity to co-morbidities such as cancer, diabetes mellitus, hypertension, cardiovascular disease, and asthma[73].

2.3.2 Leptin

Leptin the first identified adipokine, comes form the Greek word for "thin" and curbs appetite by influencing the hypothalamus[72,92]. Discovered to be the product of the OB (obese) gene, leptin studies were first conducted in mutant mice[72,92]. It was reported that mice genetically altered to not produce leptin had an insatiable appetite; high cortisol levels, did not reproduce, and grew abnormally to become severely obese[93]. However, when exogenous leptin was injected into these mice they were shown to increase locomotor and thermogeneic activity, lose weight, and eat less[93]. Leptin interacts with the leptin receptor, which is expressed primarily in the arcuate nucleus of the hypothalamus, the part of the brain that controls feeding behavior[93]. In morbidly obese individuals, leptin is found in concentrations four to six times higher than levels in a normal weight person[93]. It has been suggested that due to the positive association of increased adiposity and leptin levels, leptin may be an important link between inflammatory effects and obesity[92,93]. Leptin has been associated with obesity co-morbidities including asthma[94]. Increased leptin levels promote activation of alveolar macrophages in the lungs[95].

2.3.3 Adiponectin

Adiponectin was first identified and described over 20 years ago by the Scherer et al [96] and Maeda et al[97] groups [98]. It was once thought to be the most abundant transcript in adipose tissue and has structural homology to both Cp1[96,98-101] and TNFα[98,102]. Known

to have anti-inflammatory properties, adiponectin has been reported to have an inverse relationship with BMI[103]. Three forms of adiponectin, have been identified in plasma of humans and mice. Characterized by corresponding molecular weight low molecular weight adiponectin (LMW) weighs 67kD, middle molecular weight (MMW) 136kD, and high molecular weight 300kD, which is thought to be the most physiologically active of the three[104-107]. Produced mainly by visceral fat, Adiponectin is an adipokine insulin-sensitizing hormone and is generally associated with ant-inflammatory effects[108]. Adiponectin is a unique adipokine in that levels decrease with excess adiposity. It has been shown to induce anti-inflammatory cytokines IL-10 and the IL-1 receptor antagonist, to suppress NFκB signaling, expression of adhesion molecules, and proliferation of vascular smooth muscle cells[109-111]. Reduced adiponectin has been implicated in the pathogenesis of a variety of metabolic diseases such as insulin resistance and coronary vascular disease[112,113]. Therefore, a role in the pathogenesis of asthma is also very likely since human ASM cells express adiponectin receptors (Adipo R1 and R2)[114]. Although an epidemiological association between adiponectin and asthma has not yet been demonstrated[61] reports from recent studies suggest a relationship between asthma and adiponectin levels[115]. Shore et al found that exogenous adiponectin administration was sufficient to induce a 60% increase in serum adiponectin in a mouse model of asthma resulting in significant suppression of the OVA-induced inflammatory response and bronchial hyperreactivity[116]. In addition, adiponectin deficient mice exhibit a pulmonary vascular phenotype characterized by upregulation of E-selectin and increased perivascular inflammation[117]. This phenotype was even more apparent in older mice suggesting that prolonged adiponectin deficiency as seen in obesity may lead to a

progressive inflammatory pulmonary vascular phenotype[117]. Similarly, adiponectin knockout mice ($APN^{-/-}$) exhibit severe pulmonary arterial muscularization and exaggerated allergic airway inflammation in response to chronic OVA challenge compared to wild-type mice[118]. Further, low serum levels of adiponectin are associated with increased asthma risk[119] and deficient levels are observed during asthma exacerbations in both mouse[116] and human[94] models. High serum levels of adiponectin protect against asthma[63] although this relationship is more pronounced in women than in men[115].

2.3.4 Tumor necrosis factor alpha (TNFα)

One of the first reports linking the metabolic abnormalities of obesity with inflammation was a rodent study where diet induced obesity increased levels of the cytokine TNFα[120]. Secreted from macrophages, mast cells, eosinophils, neutrophils, smooth muscle cells, fibroblasts and epithelial cells in response to potential stimuli, TNFα can augment mast cells activation via a positive autocrine feedback loop and thereby induce its own production and release[48]. Notably, adipose tissue is also a significant source of TNFα, with increased levels found with increased adiposity[73]. The effects of TNFα are multiplex; it increases the transepithelial migration of neutrophils by increasing the expression of adhesion molecule such as ICAM-1 and VCAM-1 which promotes chemotaxis of eosinophils and monocytes, it has a role in T-cell activation, has powerful growth-promoting effects on myofibroblast proliferation and induces hyperreactivity[48,49,121,122]. Accordingly, TNFα blocking strategies were demonstrated to suppress airway inflammation in a murine model of asthma[123] Further, patients with asthma have increased levels of TNFα in bronchoalveolar fluid collected from their

airways[124]. Some studies have suggested a relationship between the severity of asthma and levels of TNFα[20]. The difficulty in controlling and treating the phenotype of obese asthmatics could be the result of elevated TNFα levels[48,49].

2.4 Asthma and Obesity Connection

2.4.1 Oxidative Stress

In addition to inflammatory stress, obesity and asthma are also associated with an increase in oxidative stress biomarkers in mice and humans[40,125,126]. This increase is most likely due to the underlying airway inflammation with recruitment and activation of inflammatory cells producing reactive oxygen species (ROS)[127]. Oxidant stress also promotes epithelial damage and inflammation as well as airway smooth muscle contraction and induction of airway hyperresponsiveness [128-130]. Oxidative stress alters the balance of inflammatory and anti-inflammatory cytokines secreted by adipose tissue; increased ROS suppresses adipose tissue expression of adiponectin and increases expression of inflammatory factors, including TNFα[44], while suppression of ROS exerts the opposite effect[131]. Thus the combination of both conditions may have synergistic effects and may contribute to the severity of asthma observed in obese subject[2].

2.4.2 Calcitriol and Adipocyte Derived Oxidative Stress

Adipose tissue is a significant contributor to systemic oxidative stress[125]. Fat accumulation stimulates NADPH oxidase 4 (Nox 4), a key factor in cellular ROS production[44] resulting in elevated ROS with excess adiposity. Adipocyte ROS production is modulated by mitochondrial uncoupling status and cytosolic Ca^{2+} signaling, both of which are regulated by calcitriol in murine and human adipocytes [132-134]. Calcitriol suppresses the expression of adipocyte uncoupling protein 2 (UCP2), resulting in greater

mitochondrial membrane potential, and stimulates cytosolic Ca^{2+} signaling, both of which lead to increased ROS production [131,133,134]. It was recently shown that physiological concentrations of calcitriol (100pM-10nM) dose-dependently stimulate ROS production in both murine and human adipocytes via both Nox 4- dependent and Nox 4-independent mechanisms and concomitantly stimulate expression and secretion of inflammatory cytokines, including TNFα, while inhibiting adiponectin expression and secretion [131,133,134]. Further, calcitriol exerts comparable effects in macrophages and stimulates macrophage-adipocyte cross talk and inflammatory response in co-culture[131].

2.5. Asthma and Obesity

2.5.1 Common Pathologies

In industrialized countries obesity and asthma are worrisome and growing public health issues[68]. The overweight and obese population are not only more likely to develop asthma but also seem to have a harder to control phenotype of asthmatic disease that is often refractory to standard treatments[60,135]. Obesity is now emerging as a widely accepted promoting factor for asthma[136]. Although, the link between the disorders is becoming undeniable, the mechanistic pathology of this association remains elusive[137]. Several factors may explain the link between obesity and asthma. The mechanical load of excess adiposity may lead to an overall reduced pulmonary function which includes a reduction in the functional residual capacity and a lower tidal volume resulting in shortened airway smooth muscle, decreased tension, and greater muscle stiffness[137, 138], possibly leading to bronchial hyperresponsiveness. However, this is unlikely to be a primary mechanism, as increased risk is found with even modest increases in body mass index. Obesity and asthma share common inflammatory and oxidative stress markers that

suggests the relationship may be immunological[2]. One potential connection between the two disorders lies in the low-grade systemic inflammation that characterizes obesity[80]. The risk for asthma increases with increasing BMI, while weight loss can improve the course of the illness. Camargo et al. found a positive association between asthma incidence and body mass index in the Nurses' Health Study II[47]. Over 85,000 women were followed for 4 years in the Nurses's Health Study II, including 1,500 who had asthma[47]. The study reported that as the definition of asthma became more specific, the positive relationship between asthma incidence and body mass index became stronger[47]. Data from the Coronary Artery Risk Development in Young Adults study, however, found only women showed a significant association between obesity and asthma[139]. Although the study followed participants over 10 years, only 310 of the 4,547 people had asthma[139]. Similarly, Canada's Nation Population Health survey also found the self-reported asthma and body mass index to be associated only in women[56]. A pitfall of this study, however, is that asthma was self-reported and not clearly defined[56]. Also, the sample size of 127 women and 49 men participants with asthma was very small compared to the over 9,000 total in the study[56]. A study conducted with Finnish participants found an increased risk in asthma in adults over 30 years of age if they were obese in their teen years[140]. Furthermore, the same study found that participants that were obese in adulthood (31 years of age) as well as in their teen years (14 years of age) were twice as likely to suffer from adult asthma compared to those that were not obese at either point[140]. A study conducted by Guerra et al found an increased risk of future asthma diagnosis in participants with a body mass index of over $\geq$28 kg/m^2 [141]. However, the study also found this association less strong in men compared to women[141]. A recent

follow-up in Finnish twins found participants with a normal body mass to have substantially lower risk of asthma development than obese participants[142]. A moderate increase in risk for asthma development in obese adults was also found in the National Health and Nutrition Examination Survey (NHANES) I Epidemiologic Follow-up Study [143]. Notable in all these prospective studies, obesity preceded asthma development[60]. Children and adolescents also exhibit an association between obesity and asthma in prospective studies[144]. The Children's Health Study found that obese and overweight boys had a significant increased risk of asthma development, but the association was not found in girls[145]. However, a study in US cities found the risk of asthma was positively associated with body mass index in female participants but not males in children 6 to 14 years of age[146].

Researchers have yet to agree upon a unified definition of the disease due to the complexity of the group of airway conditions classified under the asthma umbrella[55]. The absence of a proper definition of asthma has left different definitions used in different research projects[55]. Criteria for individual study definitions may be based on pulmonary function tests, questionnaire data, or clinical symptoms depending on the researcher[55]. In recent years, new asthmatic phenotypes have been reported in up to 50% of adult asthmatics that do not have eosinophilic inflammation, are not IgE dependent, and are not atopic[147]. Though immunological mechanisms may vary between the two, both non-atopic and atopic asthma are reported to have similar clinical symptoms[148]. New phenotypes such as the unique *obese asthmatic phenotype* have emerged based upon distinct airway inflammatory biomarkers[137].

Initial hypotheses about obesity-associated asthma did not regard airway inflammation as a physiological link between the two disorders[137,149]. However, recent studies have reported that obese asthmatics have a neutrophil dominant type of airway inflammation[70]. The emerging evidence suggesting neutrophilic-based airway inflammation in obese asthmatics is particularly pronounced in women[71,150]. One such study, found that increased neutrophilic airway inflammation and obesity are associated[151]. Obesity-associated phenotype is regarded as one of the most difficult to control and treatment refractory phenotypes of asthma[151]. Symptoms that are left uncontrolled result in more frequent acute exacerbations (asthma attacks) and a higher likelihood of lung function loss and even death[151]. It is possible that the suggested neutrophil predominant airway inflammation in obese asthmatics may explain the failure of conventional therapies[71,152,153].

2.5.2 Weight Loss in Obese Asthmatics

In support of the obesity and asthma connection, several studies have shown weight loss to improve the course of asthmatic illness[154-158]. Bariatric surgery resulted in significant improvements of clinical symptoms in morbidly obese asthmatics[154-158]. Furthermore, studies by the Macgregor et al[154] and Dhabuwala et al[157] reported that airway conditions were resolved in up to 50% of patients with asthma after receiving weight loss surgery. Quality of life in terms of asthma severity score, hospitalization, number of attacks, and medication use significantly improved in up to 90% of obese asthmatics after bariatric surgery[60]. A study conducted in Swedish obese asthmatics found patients surgically treated had substantial reductions in medication costs compared to those treated with conventional methods[159]. Studies involving obese asthmatics and

bariatric surgery are have shown promising results, however, study design, definitions of asthma, objective measures, and post surgery diet variations across trials are worth noting[60]. The absence of control groups in the obese asthmatic bariatric studies, lack of definitive measures of medication use, as well as absent bronchial reactivity and pulmonary function data have sparked some debate about interpreting results[60]. Additionally, some would argue that the improvement of the composition of diet as well as decreased energy intake after surgery might at least in part be responsible for respiratory health improvements opposed to weight loss alone[154,160].

2.5.3 Diet Interventions in Obese Asthmatics

The effects of weight loss on asthmatic symptoms have been examined in studies using non-surgical interventions in obese asthmatics such as diet modification[161]. The Stenius-Aarniala et al used a very-low-calorie diet implemented for an eight week duration in a randomized clinical trial to induce weight loss in obese asthmatics and observed favorable changes in forced expiratory volume (FEV_1), health status, dyspnea, forced vital capacity (FVC), number of acute exacerbations (asthma attacks), as well as use of rescue mediation were observed in those assigned to the very-low calorie diet in measurements taken at the end of the 8 week period and a year later[161]. On average those in the experimental diet group ended the study with a 14.5% reduction in their body weight, possibly explaining disease improvement[161]. A similar study reported a 14% body weight loss in obese asthmatic fed very-low-calorie diet for 8 weeks[162]. Participants in the experimental diet group were observed to have improvements in day-to-day variation, FEV_1, peak expiratory flow, ratio of forced midexpiratory flow rate to FVC, FVC, expiratory reserve volume, resting minute ventilation, diurnal variation and

functional residual capacity[162]. Albeit uncontrolled, another study utilized diet modification in obese asthmatics by 6 or 12-week study design based on the degree of obesity where patients were place on a 900kcal/day diet[163]. The average participant lost 17.4% of their pretreatment weight[163]. This study also introduced an exercise component that may explain the increase in body mass loss compared to studies using only diet interventions[163]. Positive changes were observed by the improvement of total lung capacity, FVC, and FEV_1, however, bronchial reactivity was unchanged[163].

2.6 Dairy Food Components, Asthma, and Adipose Tissue Inflammation

2.6.1 Dairy and Asthma

Despite controversial claims suggesting that milk or dairy consumption may trigger asthma attacks or promote mucus production, supporting scientific evidence for these claims is scant and could be found only for subgroups of patients and is confounded by claims regarding the source of the milk and underlying medical conditions [164]. In contrast, epidemiological studies suggest a protective role for dairy products, calcium and Vitamin D intake in the development of allergic disease and asthma [165-168]. A study reported by Wijga et al.[165] found that daily full cream milk and butter consumption at age 2 significantly lowered the subsequent incidence of asthma and wheezing at age 3 (3.4% vs. 5.6% and 13.7% vs. 18.4%, respectively). Waser et al [168]recently found an inverse association of farm milk consumption with asthma in schoolchildren from rural and suburban populations across Europe, and Hijazi et al.[166] reported low intake of milk as a significant risk factor for wheezing illness. Moreover, a prospective study[167] examining the association between maternal consumption of dairy products, calcium and vitamin D during pregnancy and the later risk of wheeze and eczema in the infants found a

decreased risk of infantile wheeze with higher maternal intake of total dairy products, milk, cheese, calcium and Vitamin D during pregnancy. Multiple components in dairy may be responsible for these protective effects. One study found that cis-9, trans-11-conjugated linoleic acid, a naturally occurring fatty acid in milk fat, significantly inhibited airway inflammation, airway hyperresponsiveness and mucous plugging in a murine model of asthma, which may explain the protective effects of butter and full cream milk[169]. In another study, Lee *et. al* [170] administered kefir powder through intragastric feeding to a mouse model of asthma and found significant anti-inflammatory and anti-allergic effects after treatment. Although the authors suggested that these effects might be caused by the high microbial content of kefir, it is plausible that other components such as calcium and leucine may also play a significant role; which supports the premise presently in review.

2.6.2 Dietary Calcium Suppression of Calcitriol

The ability of dietary calcium-induced suppression of calcitriol (elevated in calcium deficiency; ~70% of the obese) to attenuate oxidative and inflammatory stress was shown by Zemel et al where a significant, substantial suppression of both adipose tissue and systemic oxidative and inflammatory stress in a mouse model was reported [132,171]. The findings prompted the dietary interventions to be extended to clinical trials in overweight and obese subjects where it was found that suppressing circulating calcitriol with calcium-rich diet suppressed systemic oxidative stress by ~20%, reduced circulating TNFα and increased adiponectin by 20% ($p<0.002$) in a randomized, blinded cross-over trial design[171,172]. Although calcitriol has been recently reported to inhibit ASM proliferative response to PDGF[173], this effect occurs at concentrations that are 50-500

fold higher than physiological plasma concentrations (IC_{50}=520nM). These concentrations may be achieved in some tissues, such as the prostate, in vitamin D replete individuals and are relevant to autocrine signaling, but are not relevant to the endocrine signaling role of calcitriol proposed in Zemel *et. al* studies among others.

The two broad categories of vital calcium function are structural and signal transduction[73]. Calcium is considered to be the "universal second messenger"[73]. As secondary messenger calcium is responsible for nerve conduction/neuromuscular transmission, muscle contraction, membrane transport, hormone secretion, blood coagulation, cell growth, cell division, cell adhesion[73]. Less than one percent of skeletal calcium is available for exchange on short notice and this fraction is found in the extracellular bone fluid surrounded by "osteocytic membrane system"[73]. This is also the system involved with bone remodeling regulating bone resorption and deposition as well as osteoclast activation[73]. In the plasma, calcium exists as the ionized form (Ca^{2+}) representing ~50% of total plasma calcium[73]. The second most prominent form of calcium is protein bound composing about 45% of circulating calcium[73]. Approximately five percent of plasma calcium is complexed in the form of Ca-citrate, Ca-lactate, Ca-bicarbonate, or Ca-phosphate[73]. Ionized calcium and complexed calcium are both considered as ultrafilterable[73]. After kidney filtration of calcium, 98-99% is reabsorbed, a process that is regulated in part by the parathyroid hormone[73]. Calcium enters cells via parathyroid activated calcium channels and is transported across the cell by calbindins that are up regulated by calcitriol[73]. The Ca^{2+} ATPase regulated by parathyroid hormone and calcitriol along with the $3Na+/Ca^{2+}$ exchanger transport are responsible for calcium extrusion[72]. In the gastrointestinal tract calcium is absorbed by active transport at the

brush border by Ca-dependent-ATPase, although some transport is passive[72]. The transport is regulated by calcitriol, which is elevated when calcium intake is low and decreased once calcium intake is adequately restored[72]. Absorption is decreased with complexation such as binding to oxalic acid (from dietary sources) resulting in insoluble salt crystals[73]. Additionally, high-fiber foods and whole-grain foods high in phytic acid decrease calcium absorption in a similar way[73]. Further, long term use of medications, such as corticosteroids may also alter calcium absorption[73]. An alkai intestinal environment decreases calcium absorption due to the necessity of acidic conditions for calcium carbonate to dissolve[73]. Therefore, high protein diets that increase intestinal acidity in turn facilitate calcium absorption[73]. Once calcium carbonate is dissolved in the intestine it can then be absorbed into the blood[73]. A normal blood calcium level is ~ 2.2-2.6 mmol/L or 9-10.5 mg/dL total calcium and if levels fall below this range the parathyroid gland is stimulated to release parathyroid hormone (PTH)[73]. Parathyroid hormone increases Ca^{2+} uptake and stimulates 1α hydroxylase activity in the kidneys to convert 25OH vitamin D to 1, 25-diOH vitamin D (calcitriol)[73]. Calcitriol then stimulates Ca^{2+} uptake in the intestines as well as stimulates Ca^{2+} release from the bone to restore calcium levels to a normal range[73]. If calcium levels increase above the normal range, the thyroid gland is stimulated to release calcitonin, which stimulates Ca^{2+} deposition in the bones and reduces uptake in the intestines as well as the kidneys, thereby reducing plasma calcium levels[73].

The label of "vitamin D" is used to describe both D3 (cholecalciferol) and D2 (ergocalciferol) forms of the micronutrient [174,175]. Most terrestrial animals and living plants can produce one or both major forms of vitamin D [174]. Typically, vitamin D2 is

major form synthesized in plants due to the interaction of the provitamin D2 ergosterol and ultraviolet B light [176]. Humans can only synthesize D3 but can metabolize both D2 and D3[174]. The more active form of vitamin D is vitamin D3 that is found naturally in very few foods [177]. Natural sources of vitamin D3 are mainly fatty fish such as mackerel, sardines, and salmon[177,178]. The chief dietary sources of D3, outside of these natural sources, are fortified foods such as select cereals and milk[178]. The average person typically obtains only ten percent of their vitamin D from dietary sources[174]. For most people, the main supply of vitamin D3 is produced from the interaction of a chemical in the skin with UV radiation from the sun[175 179]. The chemical 7-dehydrocholesterol, or provitamin D3 (found in the dermal layers of the skin), forms cholecalciferol after being acted upon by UV radiation[175,179 174]. Cholecalciferol is then converted to 25-hydroxycholecalciferol (25OHD□, or calcidiol) by enzymatic action in the liver[175,179,180]. Circulating vitamin D, whether obtained from synthesis in UV-exposed skin or from the diet, is stored in adipose tissue or rapidly converted to 25OHD□[180]. After this reaction, the mitochondrial enzyme 1, α-hydroxylase (CYP27B1) is needed to convert 25OHD□ to the primary active form of calcitriol (1α, 25-dihydroxycholecalciferol) [175,179,180]. The CYP27B1 enzyme is expressed in many tissues throughout the body including the placenta and kidneys[175,179,180]. The now active calcitriol plays a critical role in calcium homeostasis in the body [174,180].

Vitamin D is a secosteroid hormone[72]. As a steroid hormone vitamin D acts via a receptor superfamily[72]. Once vitamin D binds to a receptor, it exposes a DNA binding region and subsequently increases transcription of some mRNAs and decreases transcription of others[72]. The vitamin D receptor is present in many tissues and helps to

carry out multiple functions[72]. A steroid hormone functions by binding to its counterpart where the now complexed steroid-receptor complex will translocate to the nucleus[72]. In the nucleus, the complex will bind to a DNA regulatory site and regulate transcription of multiple mRNAs and subsequent protein translation in the cytoplasm[72]. Vitamin D increases calcium and phosphorus absorption from the gastrointestinal tract (GI), increases calcium binding proteins such as calmodulin, increases calcium transport, increases renal calcium reabsorption, and increase mature osteoclasts to mobilize calcium and phosphorus from bone[72]. Free calcium in the cell [Ca^{2+}] is around 100nM while free Ca^{2+} in the blood is around 1.2 mM[73]. Calcium release from the bone is also increased when levels of calcitriol are increased[73]. When calcium levels are adequate in the body calcitonin is released, which then inhibits the release of calcium from the bone[73].

2.6.3 Leucine and Oxidative Stress

Dairy protein is rich in branched chain amino acids, including leucine. Recent data supports a role for leucine in attenuating oxidative and inflammatory stress[181,182]. We have found leucine (0.5 mM, comparable to levels achieved following a leucine rich meal) to stimulate mitochondrial biogenesis in both adipocytes and muscle cells, with an associated increase in oxygen consumption and fatty acid oxidation in both cell types[182]. This increase in energy utilization reduces the oxidative and inflammatory stress that otherwise result from nutrients and overload and obesity. The positive effects of leucine on mitochondrial metabolism have been shown to be mediated, at least in part, by sirtuin 1 (Sirt1), as leucine stimulates Sirt1 activity, while knockdown of Sirt1 inhibits leucine-stimulation of mitochondrial biogenesis and metabolism[182,183]. To test the systemic effects of leucine and calcium (in the form of dairy foods) on Sirt1, an ex vivo approach

was utilized to examine the integrated effects of dairy diets in adipocytes and skeletal muscles[183]. Serum from 20 overweight and obese subjects fed low or high dairy diets for 28 days were used to reflect the integrated systemic response to the diets and was then added to culture medium (similar to a conditioned medium approach) to treat cultured adipocytes and muscle cells for 48 hours[183]. Serum from participants fed a high, but not low dairy diet, resulted in a 40% increase in Sirt1 expression vs. baseline serum in both tissues ($p<0.01$) and a 30% increase in Sirt1 activity in adipocytes ($p<0.05$)[183]. This was associated with increased expression of adipocyte mitochondrial NADH dehydrogenase and UCP2 ($p<0.04$) and muscle UCP3, NRF1, and COX7 ($p<0.03$)[183]. Sirt1 activation of PGC1-α not only drives an increase in mitochondrial energy metabolism, but also induces antioxidant enzymes such as manganese superoxide dismutase[184] and inhibits NF-κB activity[182,184], thereby attenuating oxidative and inflammatory stress, respectively. Accordingly, leucine markedly inhibits the expression and secretion of inflammatory cytokines while increasing adiponectin expression and secretion ~two-fold in cultured adipocytes[181]. A published microarray study demonstrated that suppression of calcitriol by calcium feeding and increased leucine intake each up-regulated Sirt1-dependent signaling pathways for fat oxidation and attenuation of inflammatory response (including NFκ-B signaling) and attenuated pathways such as MAPK and JAK-STAT signaling associated with proliferations of cell cycle regulation[185].

2.7 Leucine Molecular Mode of Action

2.7.1 Sirtuins

Sirtuins are a group of nutrient sensing proteins characterized as class III deacylases that need NAD^+ molecules for deacylation processes[186,187]. Originally

characterized as a repressor of gene transcription at selected loci by acting as a chromatin silencing component the first identified sirtuin protein silent information regulator 2 (SIR2) was discovered in yeast[188]. Sirtuins are energy sensing proteins that are diet-sensitive, anti-aging, and are conserved[187]. The anti-aging properties of sirtuins defined in yeast, fruit flies, *C.elegans* and were also found to be conserved in mammals[189,190]. Three sirtuin proteins (Sirt1, Sirt6, and Sirt7) have been found to localize in the nucleus where they regulate gene transcription in response to energy changes[187]. Only recently was Sirt2 also found in the nucleus where it is distinct in function by modulating cell cycle control[191-193]. The other three sirtuins Sirt3, Sirt4 and Sirt5 are found in the mitochondria making up the total of seven mammalian sirtuins currently identified[187]. Cellular responses can be coordinated throughout the organism in times of caloric restriction due to the specific cellular compartments in which sirtuins are located[194]. Sirtuins found in the nucleus such as Sirt1, Sirt6, and Sirt7 influence gene expression by the epigenetic process of histone deacetylation[194]. Additionally, it has been found that Sirt1 influences the activities of enzymes and specific transcription factors by deacetylation[187]. Mitochondrial sirtuins Sirt3, Sirt4, and Sirt5 modulate oxidative stress in their organelle of residence and regulate activities of metabolic enzymes[195]. In response to caloric restriction (CR) mitochondrial sirtuins switch cellular processes to support mitochondrial oxidative metabolism as well as turn on genes for stress tolerance[187]. In humans, Sirt1 is activated in response to caloric restriction[196] by sensing increased NAD^+ levels, accordingly, expression is reduced in obesity[197,198]. Sirt1 functions in key metabolic tissues such as white adipose tissue (WAT), liver, heart, and skeletal muscle[187]. Gluconeogenesis is supported by Sirt1 in the liver via peroxisome proliferator-activated receptor (PPAR) γ

co-activator 1α (PGC-1α) and forkhead box O1 (FOXO1)[199]. In prolonged fasting, Sirt1 facilitates the degradation of CREB-regulated transcription co-activator 2 (CRTC2)[200]. Fatty acid oxidation is promoted in the liver by SIRT1 in response to fasting by the activation of peroxisome proliferator-activated receptor α (PPARα)[201]. Fatty acid synthesis in the liver is inhibited by SIRT1 targeting of sterol regulatory element binding protein 1c (SREBP1c) for degradation[202]. Systemic cholesterol homeostasis is controlled by SIRT1 via positive regulation of the liver X receptor (LXR)[203]. In skeletal muscle SIRT1 acts in a similar manner with the overall effects of reducing glycolysis and increasing fatty acid utilization[204]. In this tissue, AMP-activated protein kinase and SIRT1 share positive loop of regulation[187]. AMPK up regulates the gene encoding the NAD^+ synthetic enzyme nicotinamide phosphoribosyltransferase (NAMPT), thereby increasing amounts of NAD^+ necessary for SIRT1 function[205,206]. In turn, SIRT1 works reciprocally by deacetylating liver kinase B1 (LKB1) resulting in the phosphorylation and activation of AMPK[207]. Lipid utilization in muscle and liver is driven by SIRT1 activation of PPARγ in white adipose tissue (WAT) allowing the mobilization of fat to peripheral tissues[208]. Additionally, enhanced energy expenditure is increased by SIRT1 by driving white fat browning[209]. SIRT1 induced white fat browning is initiated through PPARγ deacetylation facilitating the binding of PR domain containing 16 (Prdm16)[209]. The anti-inflammatory adipokine adiponectin up regulates SIRT1 protein[210] whereas caspase I degrades SIRT1 after post high fat diet activation[211]. Sirtuins are also beneficial to the vascular system by activating endothelial nitric oxide synthase (eNOS) resulting in increased ischemic tolerance[212]. Also, SIRT1 induced PPARα activation has shown to protect against cardiac hypertrophy[213].

2.7.2 Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that regulate gene transcription[72]. Currently, there have been three identified PPAR proteins PPAR-δ, PPAR-α, and PPAR-γ[214]. Ligand activated PPAR proteins respond to dietary lipid changes by altering carbohydrate and fat metabolism genes[72]. Fatty acid derivatives and fatty acids are common ligands for PPAR family members that work in conjunction with the retinoid X receptor to form heterodimers[72]. These heterodimers allow for the binding of the complex to regulatory regions of DNA and change the rate of transcription of associated genes as a result[72]. Genes necessary for the differentiation of fibroblasts into adipocytes, lipid storage in adipocytes, and synthesis are regulated by the PPARγ nuclear receptor[72]. Adipose tissue (both brown and white) and liver are the two types of tissues where PPARγ is predominantly expressed[72]. The type 2 diabetes medication thiazolidinedione drugs activate PPARγ[72]. The genes that turn on β-oxidation of fatty acids in the liver are controlled by PPARα[72]. Present in the liver, skeletal muscle, brown adipose tissue, heart, and kidney PPARα is also needed for ketone body formation during times of fasting[72]. The most important PPAR in terms of potential drug development to treat obesity is the PPARδ, which acts in the muscle and the liver[72]. Dietary lipids stimulate PPARδ to induce transcription of genes necessary for β-oxidation as well as those that act to uncouple mitochondria to increase energy dissipation[72]. Animal studies using high-fat diets have shown that transgenic mice with an always active PPARδ to be immune to excess fat accumulation[72]. PPARδ activation results in thermogenesis, fat depletion, and weight loss by stimulating fatty acid breakdown in uncoupled mitochondria[72].

2.7.3 AMP-activated protein kinase (AMPK)

Phosphorylation by AMP-activated protein kinase (AMPK) of target proteins results in increased substrate oxidation for energy production[73]. Fatty acid synthesis is inhibited and fatty acid oxidation is activated as downstream consequences of AMPK phosphorylation[73]. Primarily, these AMPK induced responses occur in the muscle and liver and become necessary in times of nutritional deprivation[73].

2.7.4 Effects on Obesity Related Oxidative Stress

We have previously reported that calcitriol increases inflammatory and oxidative stress in murine adipocytes and muscle cells in an in vivo model while dietary calcium suppressed calcitriol and alleviated the adverse response[171,215,216]. Investigations into high calcium foods such as dairy revealed a significantly superior effect compared to calcium alone[217]. Retrospective analysis of serum samples from previous clinical trials also suggested a protective response from dairy food consumption in obese humans[217]. Observations from these studies were used to design a randomized double blind study where dairy-based smoothies significantly increased anti-inflammatory adiponectin while decreasing IL-6, monocyte chemoattractant protein 1, C-reactive protein, and TNFα inflammatory markers and 8-isoprostane $F_2\alpha$ and malondialdehyde oxidative markers in otherwise healthy overweight and obese subjects[172].

2.8 BALB/cAnNHsd Mice

2.8.1 Overview

Acquired from Harlan Laboratories, the albino stock was originally obtained by H. Bragg in 1913 and therefore called “Bragg albino” or BALB[218]. In 1923, MacDowell, in Cold Spring Harbor, NY, USA, inbred the mice. In 1932, at F26 to Snell, who added

the ‘c’ for albino[218]. The BALB/cAnNHsd line was derived from a breeding nucleus obtained from the National Institutes of Health, Bethesda[218]. The mouse line was generated to be susceptible to an allergy induced Th_2 lymphocyte response[218,219]. Susceptibility of BALB/c mice to allergic inflammation induction led to their incorporation as models in asthma studies[218,219]. In a comparison study of 7 different strains (BALB/c, BP/2, A/J, C57Bl/6, DBA/2, CBA and AKR) of mice, it was found that mice with a Th2 bias, particularly BALB/c mice, were best for human phenotypical characteristics of asthma[219]. The BALB/c mice strain is an established and well-studied model for asthma, however information on their ability to serve as an appropriate model for obesity is not as pronounced.

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Part Three

Specific Aims

Our objectives are to determine the effects of calcium, calcitriol and leucine on airway smooth muscle cell and endothelial cell function and inflammation using *in vitro* approaches in human cells and *in vivo* studies in a mouse model of asthma. The two dietary components under investigation, calcium and leucine, are most abundant in dairy foods. Accordingly, results of this study may provide a unique opportunity to use dairy products as functional foods to uncouple the relationship between obesity and asthma and to be utilized as part of a nutritional paradigm for prevention of airway inflammatory disease.

Specific Objective 1. Determine the in vitro effects of calcitriol and leucine treatment of murine adipocytes on human bronchial airway smooth muscle cells (BSMC) and lung microvascular endothelial cell (HMVEC-L) inflammation and function.

To address this objective, BSMC and HMVEC-L were initially be cultured alone and treated with calcitriol (10 nM), L- Leucine (0.5 mM), and Nitrendipine (30 nM) for 48 hours to assess direct effects of treatments on these cell types. Next, the indirect effects of above treatments on each cell-type were examined in co-culture with murine adipocytes to assess the effects of the altered adipose tissue cytokine expression in response to the treatments on BSMC and HMVEC-L function. At the end of the incubation cells and culture medium were collected for analysis. For both cell-types (BSMC and HMVEC-L), outcome measures for the inflammatory response include intracellular adhesion molecule (ICAM-1) and vascular cellular adhesion molecule (VCAM-1) as well as their cell surface markers via flow cytometry in response to the treatments in BSMCs and HMVEC-Ls. Further, we assessed the direct and indirect

(measured by using conditioned media of treated adipocytes) effects of above treatments on the adherence and of fluorescently labeled leukocytes to treated BSMCs and HMVEC-Ls under physiological flow conditions using fluorescence microscopy.

Specific Objective 2. Determine the in vivo effects of dietary calcium and leucine on mouse airway smooth muscle function, inflammation and airway hyper-reactivity.

Specific objective 2 extended these findings to an *in vivo* system by assessing the effects of dietary calcium and leucine in an established mouse model of asthma. Effects of the diets on airway inflammation (via bronchial alveolar lavage) were analyzed. Since timing of the treatment relative to the onset of airway inflammation (intervention start before sensitization vs. after sensitization) may influence the outcome, this study was conducted in separate groups of animals either prior to or following sensitization to differentiate between preventive and therapeutic effects of the diets.

Mouse model of asthma: Female mice were utilized, as asthma is more prevalent and severe in women than in men, and female mice were recently reported to exhibit more severe allergic airway inflammation than males in response to ovalbumin challenge or house dust mite exposure (1). 6 weeks old female BALB/cJ mice, purchased from The Jackson Laboratory (Bar Harbor, Me), were sensitized to ovalbumin to induce chronic allergic airway inflammation as described (2) with minor modifications. Intraperitoneal (i.p.) injections of 50 μg of Chicken egg albumin (ovalbumin [OVA]; Grade V, Sigma-Aldrich, St. Louis, MO) diluted in 0.1 ml of PBS was given on day 0, 4 and 7. Starting on day 12, animals were challenged by aerosol challenge by nebulizer delivery of 2.5% OVA diluted in PBS 3 times a week for 6 weeks. Mice assigned to nonsensitized group

received i.p injections and aerosol challenges of PBS only. Mice were euthanized 24 hours after last aerosol challenge.

Treatment groups: Animals were randomly divided into 5 different treatment groups:

- *Non-sensitized control group*: fed a high sucrose/high fat diet with sub-optimal Calcium (0.4%)
- *OVA-sensitized control group:* fed a high sucrose/high fat diet with sub-optimal Calcium (0.4%)
- *Non-sensitized high calcium group*: fed a high sucrose/high fat diet with sub-optimal Calcium (1.2%)
- *OVA-sensitized high calcium group*: fed the same diet as control but supplemented with Calcium carbonate (1.2% Ca)
- *Non-sensitized Leucine group*: fed the same diet as control but supplemented with Leucine (200% normal level)
- *OVA-sensitized Leucine group*: fed the same diet as control but supplemented with Leucine (200% normal level)
- *Non-sensitized high calcium/Leucine group*: fed the same diet as control but supplemented with calcium (1.2%) leucine (200% normal level)
- *OVA-sensitized high calcium/Leucine group*: fed the same diet as control supplemented with calcium (1.2%) and leucine (200% normal level) □

Each diet group was further divided into 2 groups, one prevention group with feeding start before sensitization initiation, and one therapeutic group with diet start 2 days before the first OVA or PBS challenge.

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Part Four

Leucine and calcitriol adipocyte treatment: Opposing influences on leukocyte adhesion to cell types of the airway

4.1 Abstract

The increase in inflammatory cytokines observed in obesity may link excess adiposity to asthmatic airway inflammation. We have previously demonstrated that 1,25 dihydroxycholecalciferol (calcitriol) and leucine modulate adipocyte and macrophage inflammatory cytokine production. With the knowledge that several of these cytokines are involved in leukocyte adhesion and infiltration into lung endothelial and bronchial smooth muscle cells of the airway in asthmatic disease, we sought to determine if calcitriol and leucine treatment of adipocytes could alter this inflammatory process. 3T3-L1 adipocytes were treated with vehicle, calcitriol, leucine or both for 48 hrs. Adipocyte conditioned medium (CM) was collected for incubation with U937 monocytes or whole blood leukocyte populations for activation marker and adhesion assays or used to assess adhesion molecule mRNA and protein expression in human lung microvascular endothelial cells (HMVEC-L) and bronchial airway smooth muscle cells (BSMC). Compared to control, calcitriol markedly increased adhesion of human monocytes to BSMC by ~7 fold, from 225 ± 14 to 1557 ± 489 cells adhering/μm^2 ($p<0.001$). Leucine CM, however, decreased monocyte adhesion to BASM by ~40% compared to control. Similarly, ICAM-1 adhesion molecule mRNA was significantly lower in leucine CM treated HMVEC-L cells as compared to calcitriol CM samples ($p<0.05$). Protein expression of ICAM-1 increased ~40% in calcitriol CM samples compared to control CM ($p<0.05$), while leucine CM samples were decreased ~48% compared to calcitriol CM ($p<0.01$). CD11b adhesion integrin expression in whole blood leukocytes treated with calcitriol CM were increased ~67% compared to control CM ($p<0.01$). Conversely, leucine CM treated leukocytes expression was reduced by ~40% compared to control CM

($p<0.01$). Data from this study suggest opposing roles of leucine and calcitriol in obesity associated airway inflammation.

4.2 Introduction

Over the past several decades the prevalence of asthma has increased dramatically in urbanized nations such as the United States (1-3). Upwards of 300 million people suffer from asthma worldwide with over 24 million currently living in the US (1, 3). Accompanying asthma as a public health issue is the upsurge of global obesity incidence (4). With the observed parallel growth of obesity and asthma cases, an increasing body of evidence suggests a positive relationship between the two disorders (5). Obesity is now widely recognized as a key risk factor for asthma where relative risk has been shown by prospective studies to increase with increasing body mass index (BMI) (5, 6). In support of this connection, several studies have reported increased prevalence, incidence, severity, and decreased control of asthma in obese individuals (7-11). There is increasing proof that obesity is a prompting factor for asthma, however, the mechanistic link between obesity and asthma remains elusive (10, 12). One hypothesis to explain the connection lies in the increased systemic inflammation observed with excess adiposity that is now a hallmark of obesity (13, 14, 15). It has been reported that adipokines and cytokines derived from the adipose tissue of obese asthmatics may have a synergistic adverse effect on the airways (16). The defined roles of most of these intermediaries in the development of asthmatic disease have not been demarcated; however, the alteration of anti-inflammatory adiponectin (decreased in obesity) and pro-inflammatory TNFα (increased in obesity) adipokines has been described as potential participants in the pathogenesis of asthmatic disease (17-22).

Airway inflammation opulent in leukocytes (eosinophils, neutrophils, monocytes) is frequently used to characterize asthma phenotypes (23-25). The adipokine TNFα has been implicated in asthmatic airway inflammation in both *in vitro* and *in vivo* models (26-28). The effects of TNFα are multiplex; it increases the transepithelial migration of neutrophils by increasing the expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1), which promotes chemotaxis of eosinophils and monocytes (26-28). Polymorphonuclear cell (PMNs) leukocytes especially eosinophils and neutrophils are critical pathogenic components in asthmatic disease by infiltrating the airways (29). The cytokine-induced increase of leukocyte activation integrins and chemotactic guidance of chemokines recruit PMNs from the blood to the site of inflammation in the airways (29). The interaction between intracellular adhesion molecules (ICAM-1) on vascular endothelial cells and CD11b (β_2-integrin) on leukocytes secure adhesion of PMNs to endothelial cells (29). Contrary to the pro-inflammatory contribution of TNFα to airway inflammation the adipokine adiponectin appears to be beneficial in airway disease (30-32). Adiponectin has been shown to induce the anti-flammatory cytokines IL-10 and the IL-1 receptor antagonist, to suppress NF-κB signaling, and to suppress the expression of adhesion molecules (30-32).

In addition to inflammatory stress, both obesity and asthma are also associated with an increase in oxidative stress biomarkers in mice and humans (33-35). Oxidative stress alters the balance of inflammatory and anti-inflammatory cytokines secreted by adipose tissue; increased reactive oxygen species (ROS) suppresses adipose tissue expression of adiponectin and increases expression of inflammatory factors, including

TNFα (36), while suppression of ROS exerts the opposite effect. Adipose tissue is a significant contributor to systemic oxidative stress. Our lab, and others has shown adipocyte ROS production to be modulated by mitochondrial uncoupling status and cytosolic Ca^{2+} signaling, both of which are regulated by calcitriol (vitamin D_3) in murine and human adipocytes (37-39). Calcitriol (unregulated in calcium deficiency and obesity) suppresses expression of adipocyte uncoupling protein 2 (UCP2), resulting in greater mitochondrial membrane potential, and stimulates cytosolic Ca^{2+} signaling, both of which lead to increased ROS production (37-40). We have recently shown that calcitriol dose-dependently stimulates ROS production in murine and human adipocytes and concomitantly stimulates the expression and secretion of inflammatory cytokines, such as TNFα, while inhibiting adiponectin expression and secretion (37-40). Consequently, we evaluated the ability of dietary calcium-induced suppression of circulating calcitriol to attenuate oxidative and inflammatory stress and found significant suppression of both adipose tissue and systemic oxidative and inflammatory stress in a mouse model (37, 41). These studies also began to suggest a role for the branched chain amino acid leucine in attenuating oxidative and inflammatory stress as well (43, 44). Leucine was shown to stimulate mitochondrial biogenesis, oxygen consumption, and fatty acid oxidation in adipocytes and muscle cells (43, 44). The increased energy utilization due to leucine treatment in these cell types resulted in reduced oxidative and inflammatory stress that otherwise would result from nutrient overload and obesity (43, 44). From this previous data we hypothesize that the alteration in adipocyte cytokine production by leucine and calcitriol treatment will have opposing affects on airway inflammation. We used human microvascular endothelial cells of the lung (HMVEC-L) and bronchial airway smooth

muscle cells (BSMC) to assess adhesion molecule expression and inflammatory markers. Leukocytes from whole blood were used to observe CD11b integrin and activation marker expression and subsequent adhesion to either lung endothelial or bronchial airway smooth muscle cell monolayers.

4.3 Materials and Methods

4.3.1 Adipocyte Conditioned Medium (CM) Generation

3T3-L1 preadipocytes (ATCC, Manassas, VA) were incubated at a density of 8000 cells/cm^2 (10 cm^2 dish) and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) containing 10% Fetal Bovine Serum (FBS) (Gibco, Grand Island, NY) and antibiotics (1% penicillin/streptomycin) at 37° C in 5% CO_2 in air. Confluent preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1; v/v) medium supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and antibiotics (1% penicillin/streptomycin). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium (10% FBS, DMEM, 1% penicillin/streptomycin). Cultures were re-fed every 2-3 days to allow 90% cells to reach full differentiation. Before treatments, cells were incubated in low serum medium (0.2% FBS, DMEM, 1% penicillin/streptomycin) overnight and then washed with fresh medium, re-fed with (2% FBS, DMEM, 1% penicillin/streptomycin) medium containing the different treatments (vehicle, 0.5 mM leucine, 10 nM calcitriol, 0.5 mM leucine and 10 nM calcitriol combination) all obtained from Sigma (St. Louis, MO) and incubated at 37 °C in 5% CO_2 for 48 hr before conditioned media collection. Adipocyte conditioned medium was used immediately or stored at -80°C until needed.

4.3.2 Human Lung Microvascular Endothelial Cells (HMVEC-L)

HMVEC-L cells were maintained following the distributor's protocol (Lonza, Walkersville, MD CC-2527), in endothelial basal medium-2 (EBM-2) (Lonza, Walkersville, MD CC-3156) supplemented with endothelial growth medium (EGM-2) MV BulletKit from (Lonza, Walkersville, MD CC-3202) at 37°C and 5% CO_2, until achieving a 70%-90% confluence. The cells were used at passages 5-9 for assays.

4.3.3 Bronchial Airway Smooth Muscle Cells (BSMC)

BSMCs (Lonza, Walkersville, MD CC-2576) were incubated at a density of 8000 cells/cm^2 (10cm^2 dish) and grown in smooth muscle cell basal medium (SmBM-2) and corresponding bullet kit smooth muscle cell growth medium (SmGM-2) (Lonza, Walkersville, MD CC-3182) at 37°C and 5% CO_2. BSMC were utilized for assays between passages 4-6.

4.3.4 U937 Monocyte Cell Line (U937s)

U937 monocyte cells were obtained from ATCC (Manassas, VA, USA) and suspensions were cultured in T-75 cm^2 flasks with RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco, Grand Island, NY) and antibiotics (1% penicillin/streptomycin) at 37°C and 5% CO_2. Cell density was maintained between 1 X 10^5 and 2 X 10^6 viable cells/mL. Fresh medium was added every 3-4 days depending on the number of cells.

4.3.5 Adipocyte-Bronchial Airway Smooth Muscle Co-Culture

3T3-L1 mature adipocytes and BSMCs were co-cultured using transwell inserts with a 0.4 μm porous membrane (Corning Inc., Corning, NY) to separate adipocytes and BSMCs, as previous described with adipocyte-myocyte interactions (45). Each cell type

was grown independently in transwell plates and following differentiation; inserts containing adipocytes were transferred to bronchial smooth muscle cell plates. The cells are then incubated in DMEM-medium with antibiotics containing the indicated chemical treatments (vehicle, 0.5 mM leucine, 10 nm calcitriol, 0.5 mM leucine and 10 nM calcitriol combination, 30 nM nitrendipine (Sigma, St. Louis, MO), 10 nM calcitriol and 30 nM Nitrendipine combination) and incubated at 37°C in 5% CO_2 for 48 hr, after which cells in the lower well are harvested for further analysis and media collected for subsequent experiments.

4.3.6 RNA Extraction

The Ambion ToTALLY RNA isolation kit (Ambion, Inc., Austin, Tex., USA) was used to extract total RNA from cells according to the manufacturer's instruction. The concentration, purity and quality of the isolated RNA were assessed by measuring the 260/280 ratio (1.8-2.0) and 260/230 ratio (close to 2.0) by using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Del. USA).

4.3.7 Gene Expression

Expression of HMVEC-L and BSMC 18S, intracellular adhesion molecule (ICAM-1), vascular cellular adhesion molecule (VCAM-1), and tumor necrosis alpha-receptor 1 (TNFαR1) were measured via quantitative real-time PCR using an ABI 7300 Real-Time PCR system (Applied Biosystems, Branchburg, NJ) with a TaqMan core reagent kit. All primers and probe sets were obtained from Applied Biosystems TaqMan Assays-on-Demand and utilized accordingly to manufacturers instructions. Pooled RNA from each cell type was serial-diluted in the range of 0.0156-50 ng and used to establish a standard curve; total RNA for each unknown samples was also diluted in this range. RT-

PCR reactions were performed according to the instructions of the ABI Real-Time PCR system and TaqMan Real Time PCR Core Kit. Expression of each gene of interest was then normalized using the corresponding 18S quantification. Data for each gene is presented as a ratio of 18S.

4.3.8 Laminar Flow Adhesion Assay

BSMCs were cultured as previously described until approximately 90% confluent. Cells were then removed from the T-75 cm^2 flask with trypsin (0.25%)/EDTA (0.1 μM), and centrifuged at 200 x g for 5 min. Cells were resuspended with 3 mL of cells culture media and counted using an improved Neubauer cell counting chamber with trypan blue staining to assess viability. Cells were seeded onto 40 mm glass coverslips at a final concentration of 2.5 $x10^5$ cells/mL and incubated at 37°C, 5% CO_2 for 12-24 hrs until approximately 90% confluent. Cells were washed 3 times with phosphate buffered saline (PBS) and fluorescently labeled with Calcein Am (Invitrogen, Carlsbad, Ca)(1 μg/mL) for 30 min. at 37° C, 5% CO_2. Afterwards, the coverslips were washed 3 times with PBS to remove excess Calcein AM.

U937 cells (monocyte line) were centrifuged at 200 x g for 5 minutes. Cells were resuspended in 3 mL of FBS free, phenol red free medium and counted as previously described. Viability of U937 cells was >90% for all assays. U937 cells were adjusted to a final concentration of 1.0 x 10^6 cells/mL. The cells were fluorescently labeled with 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)(Sigma, St. Louis, MO) at a final concentration of 4.16 μg/mL for 20 min. at 37°C, 5% CO_2. After labeling, the cells were topped off with FBS free, phenol red free RPMI 1640 medium and centrifuged at 200 x g for 10 minutes. The supernatant was removed and the labeled

U937 cells were resuspended with 3mL of either (vehicle, 0.5 mM leucine, 30 nM nitrendipine, 10 nM calcitriol or calcitriol and nitrendipine combination) adipocyte-BSMC co-culture medium (1.0 x 10^6 cells/mL final concentration) or by the same chemicals and concentrations diluted in RPMI 1640 medium for direct treatments.

4.3.9 U937 Monocyte-BSMC Adhesion Assay

This assay was modified from a previously described method (46). Briefly, a coverslip containing a monolayer of Calcein AM labeled BSMCs were assembled in a FCS2 microscopy stage mounted perfusion system (Bioptechs Inc, Butler, PA) containing 0.5 mm gaskets. FBS free, phenol red free RPMI 1640 cell culture media (Gibco, Grand Island, NY) was perfused across the BSMC monolayer at a flow rate of 0.5 mL/min (shear rate=132.9 s^{-1}) for 10 min. Afterwards, the pump was stopped and the affluent tubing was placed in a 12 x 75 mm tube containing the prepared U937 cells in adipocyte-BSMC co-culture or directly supplemented media. The pump was restarted and ran for 5.5 min., which was the optimized time to allow fluid to flow through the entire system to the effluent end. The pump was then stopped, and the effluent end of the tubing was placed back into the original 12 x 75 mm tube, allowing for a continuous, recycled perfusion. The pump was then restarted and ran for 1 hr. Afterwards, unbound U937 cells were washed off for 10 min with FBS-free, phenol red-free RPMI 1640 media. For comparative purposes, a perfusion with the control adipocyte-BSMC co-culture media was ran every day prior to running a perfusion with a given treatment group. This allowed for each treatment group perfusion (leucine, calcitriol, nitrendipine, or calcitriol and nitrendipine combination) to be normalized to the control adipocyte-BSMC co-culture or vehicle direct supplemented media each day.

4.3.10 Microscopy and Image Analysis

Microscopy was performed on a Nikon Eclipse Ti-E Epi-fluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with an automated stage and a 20 X objective. A 3 X 3 large image scan was taken in each of 5 random fields by multi-channel capture (channel 1: excitation/emission=488/517 nm, channel 2: ex/em 550/567 nm). Image analysis was performed using Nikon Elements 3.1 software (Nikon, Melville, NY). Binary thresholding was individually applied to both cell types to exclude non-specific staining, generating a percent area occupied by fluorescence, which has been shown to be a direct correlation to the cell count (47). Results are presented as the ratio of percent area occupied by fluorescence (U937: BSMC).

4.3.11 Phlebotomy

Blood draws were approved from an ethical standpoint by the Institutional Review Board at the University of Tennessee (IRB #8961 B). Subjects interested in donating blood were required to meet standard inclusion criteria guidelines that were out lined in the screening form. Blood was drawn using a 19-guage butterfly needle equipped with a vaccutainer connection and 10-30 mL of blood was collected each time into sodium heparin vaccutainer tubes (BD Biosciences; San Jose, CA). Blood was immediately used for all assays described below.

4.3.12 Whole Blood Leukocyte CD11b Expression by Flow Cytometry

Whole blood samples were collected and 500 µl of each sample was placed into a 2 mL eppendorf tube. Previously generated adipocyte CM (vehicle, leucine, calcitriol, leucine and calcitriol combination) in the amount of 500 µl was added to the 2 mL tube and incubated with the blood at 37°C, 5% CO_2 for 1 or 2 hours. After incubation, the

whole blood and adipocyte CM mixture was fixed in 2% paraformaldehyde for 10 min. The tubes were then topped off with PBS and centrifuged at 200 X g for 5 min. Cell pellets were resuspended in 100 µl of primary labeling mixture containing 1 µg of mouse, anti-human CD11b monoclonal antibody (abcam, Cambridge, MA) diluted in 1 mL of PBS. The primary antibody was incubated at 25°C for 1 hr. The cells were washed with PBS and centrifuged as previously described. A secondary antibody conjugated to Alexaflour 488 (1µg of goat, anti-mouse IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted in 1 mL of PBS and subsequently incubated with the cells for 30 min at 25°C. Cells were washed, centrifuged and resuspended in PBS. Flow cytometry was performed on an Accuri C6 Cytometer (Accuri, Ann Arbor, MI). Twenty thousand events were collected and a marker gate was set on the isotypic control. The same gate was applied to the CD11b stained samples generating a percent of cells that are positive for CD11b expression.

4.3.13 ICAM-1 or CD54 Expression Flow Cytometry

HMVEC-Ls were cultured as previously described. Cells (1.5×10^5) were seeded in each well of a 6 well plate and grown at 37°C, 5% CO_2 for 24-48 hrs until approximately 90% confluent. HMVEC-L cells were washed with PBS and incubated with 2 mL of the various adipocyte CM (vehicle, leucine, calcitriol, leucine and calcitriol combination) for 48 hrs at 37°C, 5% CO_2. After incubation, the cells were washed 3 times with PBS and removed from the wells with trypsin (0.25%)/EDTA (0.1 µM). Cells were centrifuged at 250 X g for 5 min and resuspended in 2% paraformaldehyde for 10 min fixation. The tubes were topped off with PBS and centrifuged as previously described. Cell pellets were resuspended in 100 µl of PBS and incubated with 1 µg of

mouse, anti-human CD54 monoclonal antibody (Beckman Coulter, Brea, CA) for 1 hr at 25°C. Cells were washed with PBS and centrifuged as previously described. Cell pellets were resuspended in 100 µl of PBS and incubated with 1µg of goat, anti-mouse Alexa 488 (IgG1) secondary antibody (Invitrogen, Grand Island, NY) for 45 min at 25°C. Cells were washed with PBS and centrifuged as previously described. Pellets were resuspended in 300 µl PBS and analyzed by flow cytometry as described above.

4.3.14 Statistical Analysis

Results are shown as mean (±SEM) values. Groups were compared by a one-way analysis of the variance of the means (One-way ANOVA). Data was tested for equal variances (Levene's Test) and normality before ANOVA analysis. Differences among groups determined by Tukey's HSD. A *P* value of less than 0.05 was regarded as indicative of a significant difference.

4.4 Results

4.4.1 ICAM-1/CD54 mRNA expression in HMVEC-L

HMVEC-L cells were harvested and mRNA extracted following 48 hr. incubation with treated adipocyte CM. ICAM-1 mRNA was normalized to 18S providing ICAM-1/18S ratio that was compared among groups. Leucine CM treated endothelial cells had significantly reduced ICAM-1 mRNA levels compared to calcitriol CM treated cells ($p<0.05$). Leucine in combination with calcitriol CM significantly reduced the amount of ICAM-1 mRNA in HMVEC-L cells compared to lung endothelial cells treated with calcitriol alone ($p<0.05$) (Figure 1).

4.4.2 ICAM-1/CD54 protein expression in HMVEC-L via Flow Cytometry

Calcitriol CM treated HMVEC-Ls had significantly higher levels of ICAM-1 protein expression compared to control CM treated cells ($p<0.05$). Leucine CM treated lung endothelial cells, however, had significantly lower levels of ICAM-1 protein expression calcitriol CM treated ($p<0.01$) cells. The addition of leucine to calcitriol CM significantly reduced the levels of ICAM-1 protein expression compared to calcitriol CM treated cells alone ($p<0.05$) (Figure 2).

4.4.3 Leukocyte Activation Marker CD11b Expression Using Flow Cytometry

CD11b expression in leukocytes treated with calcitriol adipocyte CM were increased by approximately 67% compared to control adipocyte CM treated PMNC leukocytes ($p<0.01$) (Figure 3). Conversely, leucine adipocyte CM treated leukocytes were observed to have approximately a 40% reduction in CD11b expression compared to control ($p<0.01$). Upon the addition of leucine to the calcitriol adipocyte CM treated leukocytes there was a significant reduction in the protein expression of the activation integrin CD11b protein expression ($p<0.05$).

4.4.4 Laminar Flow Adhesion Assay U937 Monocytes-BSMC Monolayer

Calcitriol direct treatment, without adipocyte exposure, markedly increased adhesion of U937 monocytes to BSMC by approximately 7 fold, from 225 ± 14 to 1557 ± 489 cells adhering/μm^2 ($p<0.001$) (Table 4-1). Notably, this effect is not mediated by calcium signaling, as inhibition of Ca^{2+} influx with nitrendipine did not reverse this effect, and in

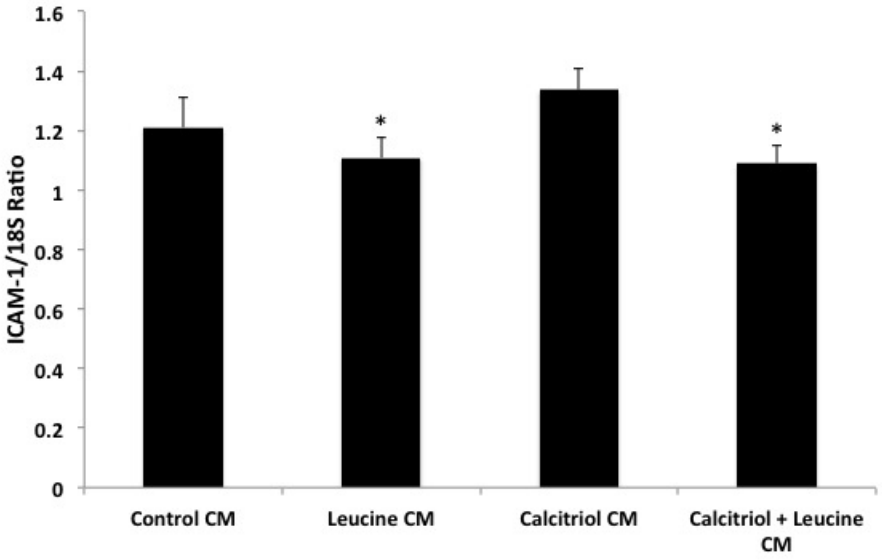

Figure 4-1: Effects of various adipocyte CM on ICAM-1 mRNA in HMVEC-L cells. Values are expressed as mean ± SEM, (n=6). * Significantly different from calcitriol CM, ($p < 0.05$).

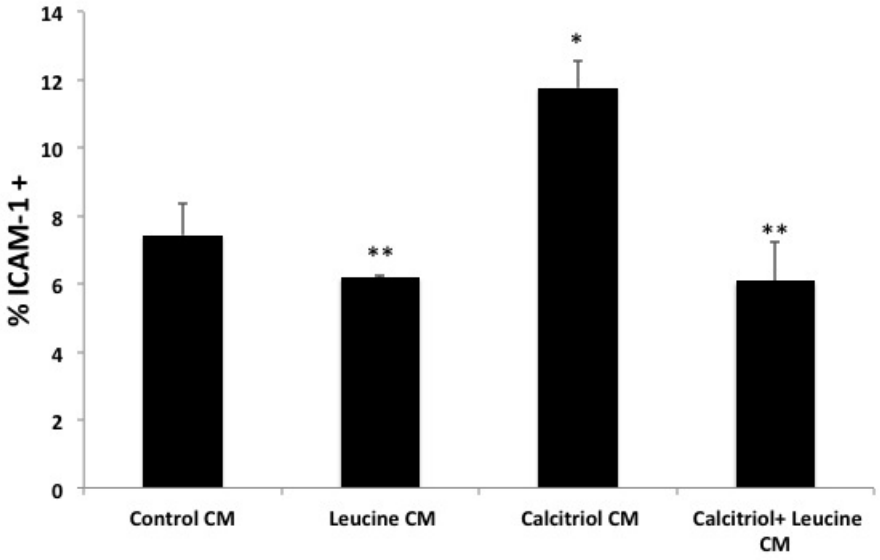

Figure 4-2: Effects of various adipocyte CM on ICAM-1 protein expression in HMVEC-L cells. Values are expressed as mean ± SEM, (n=6). * Significant different from control CM, ($p < 0.05$). **Significantly different from calcitriol CM, ($p<0.05$).

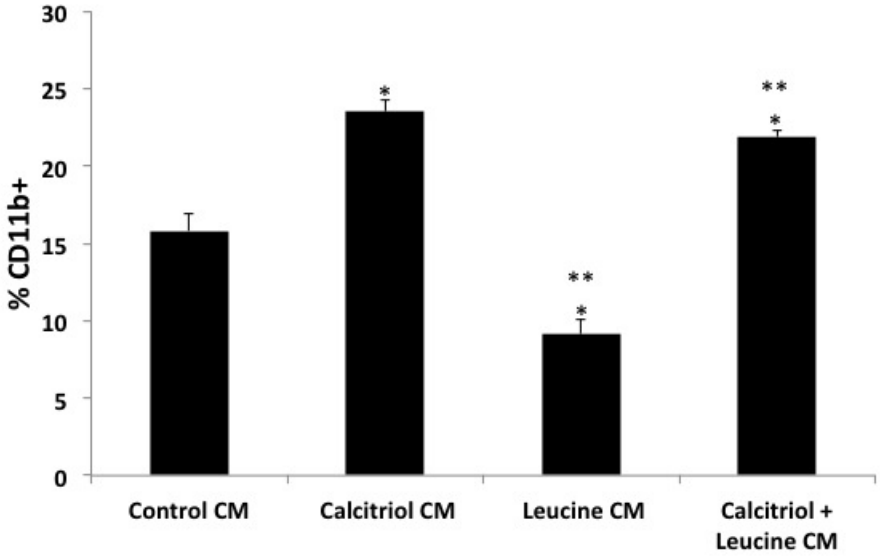

Figure 4-3: Figure 4-2: Effects of various adipocyte CM on CD11b protein expression in whole blood leukocytes. Values are expressed as mean ± SEM, (n=6). * Significant different from control CM, ($p < 0.05$). **Significantly different from calcitriol CM, ($p<0.05$).

Table 4-1: U937-BSMC Adherence (Direct Treatments)

	Direct Treatment U937-BSMC Adherence				
	Control	0.5 mM Leucine	10 nM Calcitriol	30 nM Nitrendipine	10 nM Calcitriol + 30 nM Nitrendipine
U937s/ μm^2	225 ± 14	885 ± 1	1557 ± 489	1023 ± 141	2384 ± 247

fact resulted in further stimulation of adhesion. Nitrendipine increased monocyte adhesion to BSMCs ($p<0.05$). The addition of a combined calcitriol and nitrendipine caused a significant increase in monocyte adhesion to BSMCs ($p<0.01$). There was also a slight increase in U937 monocyte adhesion to BSMCs with direct leucine treatment ($p<0.05$).

Adipocyte-BSMC co-culture studies demonstrate a similar effect of calcitriol on adherence ($p<0.05$), but in contrast to the direct effects of the chemical treatments, leucine treatment resulted in an approximate 50% reduction in adhesion of U937 human monocytes to BSMCs ($p<0.001$). Similar to the direct effects of the chemical treatments, nitrendipine exerted no effect, indicating the observed effects of calcitriol are not mediated by Ca^{2+} signaling. The data are summarized in Figure 4 A&B (direct) and Figure 5 (adipocyte-BSMC co-culture).

4.5 Discussion

Leucine and calcitriol adipocyte CM appear to have opposing effects on leukocyte activation as measured by CD11b, ICAM-1 protein expression, and monocyte-BSMC adhesion. Adhesion molecule protein expression of ICAM-1 was increased by calcitriol CM treatment of HMVEC-Ls whilst leucine decreases this effect compared to control. Leucine CM appears to initiate an anti-inflammatory response in leukocytes as indicated by the reduction in the CD11b activation marker compared to control. On the contrary, calcitriol adipocyte CM increased leukocyte activation indicated by the significant increase in the activation marker CD11b compared to control. However, leucine does

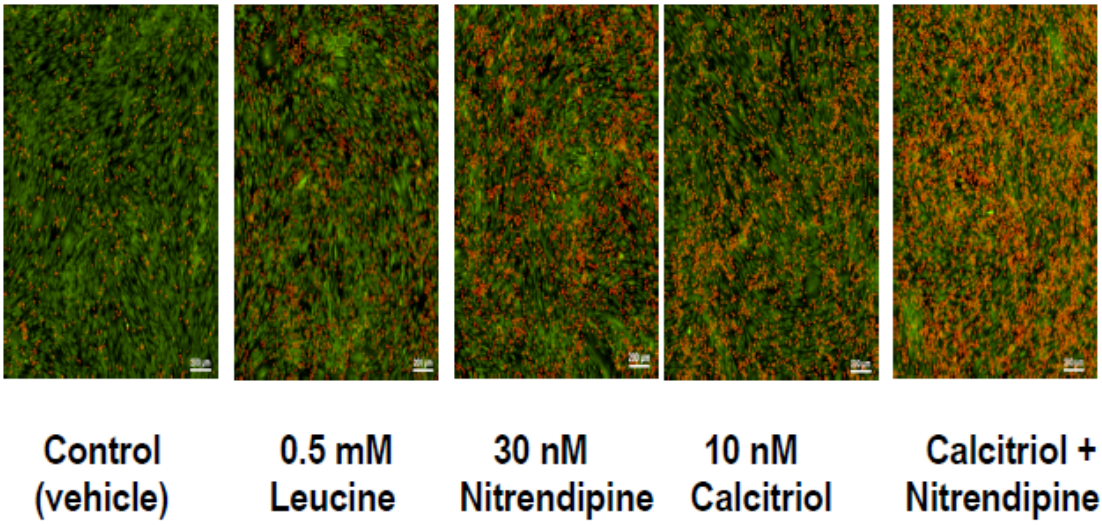

Figure 4-4 A: Direct Treatment U937 monocyte adherence to BSMC monolayer

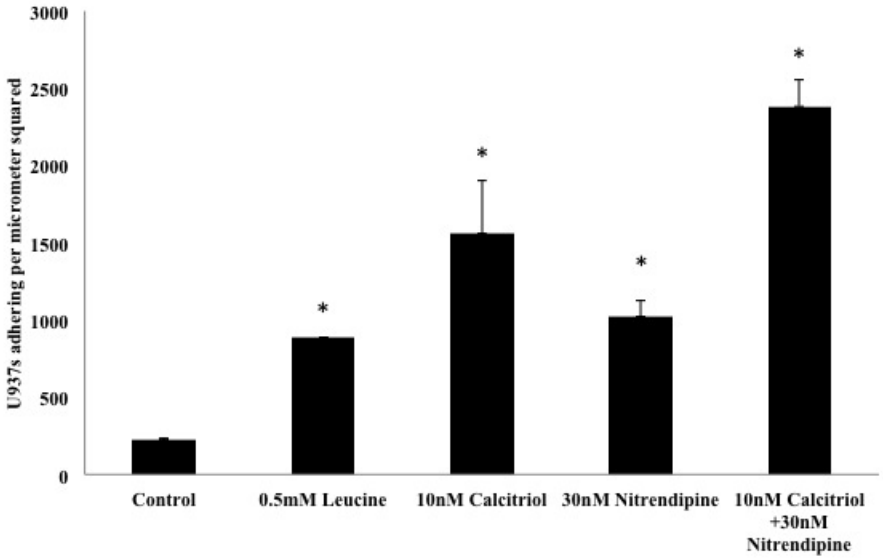

Figure 4-4 B. U937 cells per micrometer squared area on BSMC monolayer observed with the various direct chemical treatments. Values are expressed as mean ± SEM, (n=6). * Significantly different from control CM, ($p < 0.05$).

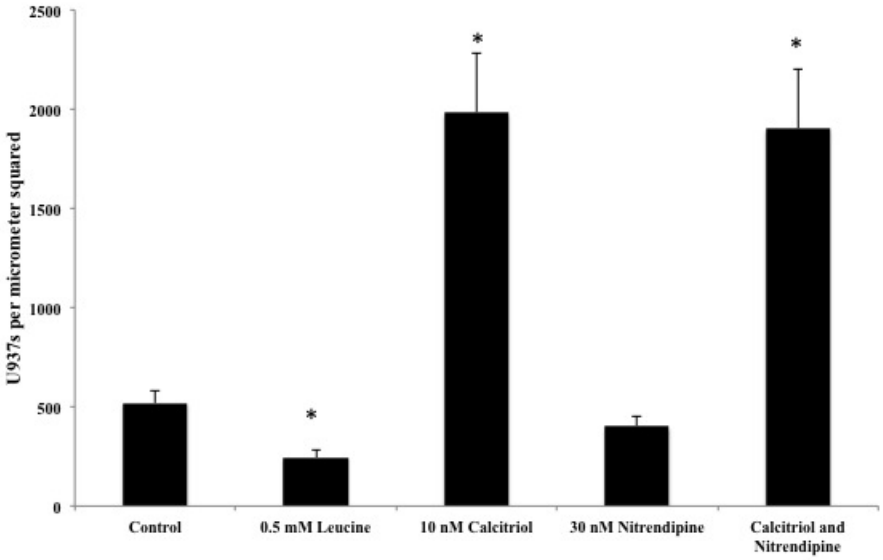

Figure 4-5: U937 cells per micrometer-squared area adhered to BSMC monolayer treated with adipocyte co-culture conditions. Values are expressed as mean ± SEM, (n=6). *Significantly different from control CM, ($p < 0.05$).

appear to ameliorate these effects of calcitriol as shown by the decrease in leukocyte CD11b expression when adipocyte CM contained both calcitriol and leucine versus calcitriol alone.

From previous studies, we know that leucine treatment of adipocytes increases the anti-inflammatory adiponectin and decreases the pro-inflammatory TNFα (37-45). However, the exact mechanism of how leucine adipocyte CM decreases adhesion molecule expression, leukocyte integrin expression, and subsequent adhesion is unclear. Leucine, when applied directly to U937 monocytes and BSMCs, increased leukocyte adhesion. While this appears counterintuitive based on our hypothesis, it is important to emphasize that this is a direct effect of leucine (no exposure to adipocyte metabolism), while the more physiologically relevant effect is found in the co-culture experiment where monocytes and BSMCs were exposed to adipocyte CM. It is likely that the differences in adipocyte cytokine production among the various treatments (control, leucine, calcitriol) are responsible for these functional responses. The data from this study suggest that leucine could attenuate leukocyte activation, adhesion molecule expression, and leukocyte adhesion resulting from excess adiposity related inflammation.

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Part Five

Leucine and calcium supplemented high fat diets and airway inflammation in a murine model of asthma

5.1 Abstract

The increase in inflammatory cytokines observed in obesity may link excess adiposity to asthmatic airway inflammation. We have previously demonstrated that 1, 25 dihydroxycholecalciferol (calcitriol) and leucine modulate adipocyte and macrophage inflammatory cytokine production. Since, several of these cytokines are also involved in asthmatic disease, we sought to determine if high fat diets supplemented with calcium, leucine, or both would alter airway inflammation in an established murine model of asthma. Diet-induced obese female BALB/c mice sensitized and challenged with ovalbumin (OVA) were fed high fat diets with no supplementation, high dose calcium (1.2%), leucine (24g/kg; 200% normal level), or calcium (1.2%) in combination with leucine (24g/kg; 200% normal level) over a six-week period. Mice fed a diet supplemented with calcium and leucine exhibited a significant decrease in eosinophil leukocytes present in lung bronchoalveolar lavage fluid (BALF) as compared to control diet animals ($p<0.05$). Our data suggests that calcium and leucine may provide a synergistic attenuation of lung leukocyte infiltration and subsequent airway inflammation in obesity-associated asthma.

5.2 Introduction

Asthma and obesity have increased in prevalence over the past thirty years in a parallel fashion (1-4). Epidemiological, longitudinal, and prospective studies suggest that the positive relationship between obesity and asthma may be a casual one (5-8). However, the mechanistic link between the two is still elusive. As asthma and obesity share many of the same inflammatory cytokines and oxidative stress markers a likely connection may be immunological (9-11). It is widely acknowledged that chronic low-

grade systemic inflammation is a hallmark of obesity and this inflammation along with oxidative stress may work synergistically to adversely modify the airways (11-19). Oxidative stress alters the balance of inflammatory and anti-inflammatory cytokines secreted by adipose tissue. Increased reactive oxygen species (ROS) suppresses adipose tissue expression of adiponectin and increases expression of inflammatory factors, including TNFα (20), while suppression of ROS exerts the opposite effect. Adipose tissue is a significant contributor to systemic oxidative stress. Our lab and others have shown adipocyte ROS production to be modulated by mitochondrial uncoupling status and cytosolic Ca^{2+} signaling, both of which are regulated by calcitriol (1,25 dihydroxycholecalciferol) in murine and human adipocytes (21-23). Calcitriol (elevated in calcium deficiency and obesity) suppresses expression of adipocyte uncoupling protein 2 (UCP2), resulting in greater mitochondrial membrane potential, and stimulates cytosolic Ca^{2+} signaling, both of which lead to increased ROS production (21-24). We have recently shown that calcitriol dose-dependently stimulates ROS production in murine and human adipocytes and concomitantly stimulates the expression and secretion of inflammatory cytokines, including TNFα, while inhibiting adiponectin expression and secretion (21-24). Consequently, we evaluated the ability of dietary calcium-induced suppression of circulating calcitriol to attenuate oxidative and inflammatory stress and found significant suppression of both adipose tissue and systemic oxidative and inflammatory stress in a mouse model (21, 25). Data from these studies also support a role of the branched chain amino acid leucine in attenuating oxidative and inflammatory stress (26, 27). Leucine was shown to stimulate mitochondrial biogenesis, oxygen consumption and fatty acid oxidation in adipocytes and muscle cells (26, 27). The

increased energy utilization due to leucine treatment in these cell types resulted in reduced oxidative and inflammatory stress that otherwise would result from nutrient overload and obesity (26, 27). Consequently, we postulated that dietary calcium and leucine would exert synergistic and beneficial effects on adipocyte cytokine secretion in diet induced obese mice leading to attenuation of airway inflammation in a murine model of asthma.

5.3 Materials and Methods

5.3.1 Animals and Diets

Six week old female BALB/c mice (Harlan Laboratories, Indianapolis, IN) were fed a high fat-diet (fat increased to 45% of energy) to induce obesity (Research Diets D12451) (Table 5-1) with either no supplementation (Control Diet), calcium raised to 1.2% (High Calcium Diet), leucine 24g/kg; 200% of normal level (Leucine Diet), or the combination of the calcium 1.2% and leucine 24g/kg (High Calcium and Leucine Diet). Animals were divided into two phases (Table 5-2 A-D) (40 animals in each) and were randomly assigned to one of the four diets on either two days before the first intraperitoneal (i.p.) injection (prevention phase) or were fed Control diets before being randomly assigned to stay on Control diet or move to a supplemented diet on Day 10 (treatment phase). Within each phase and each diet the animals were divided so that OVA allergic airway inflammation would sensitize half of the mice and the other mice would serve as a PBS vehicle control animals. The animals (four/cage) were housed in

Table 5-1: Mouse Diet Composition

	Diets			
	Control (Control)	*High Calcium (HC)*	*Leucine 200% (Leucine)*	*High Calcium +Leucine 200% (HC + L)*
	(0.4% Ca)	*(1.2% Ca)*	*(0.4% Ca)*	*(1.2% Ca)*
Ingredient (gm)				
Casein, 80 Mesh	200	200	200	200
L-Cystine	3	3	3	3
L-Leucine	0	0	18	18
Corn Starch	72.8	72.8	72.8	72.8
Maltodextrin 10	100	100	100	100
Sucrose	172.8	172.8	172.8	172.8
Cellulose, BW200	50	50	50	50
Soybean oil	25	25	25	25
Lard	177.5	177.5	177.5	177.5
Mineral Mix S10026	10	10	10	10
Calcium Carbonate	10	30	10	30
Potassium Citrate	6.2	6.2	6.2	6.2
Potassium Phosphate	13	13	13	13
Vitamin Mix V10001	10	10	10	10
Choline Bitartrate	2	2	2	2
Macronutrients (gm/kg diet)				
Protein	203	203	203	203
Carbohydrate	355	355	355	355
Fat	203	203	203	203
Fiber	50	50	50	50

Table 5-1, Continued.

	Diets			
	Control (Control) *(0.4% Ca)*	*High Calcium (HC)* *(1.2% Ca)*	*Leucine 200% (Leucine)* *(0.4% Ca)*	*High Ca + Leucine 200% (HC + L)* *(1.2% Ca)*
Macronutrients (kcal/kg diet)				
Protein	811	811	811	811
Carbohydrate	1420	1420	1420	1420
Fat	1826	1826	1826	1826
Total	4057	4057	4057	4057
Macronutrients (kcal%)				
Protein	20	20	20	20
Carbohydrate	35	35	35	35
Fat	45	45	45	45
Total	100	100	100	100
Calcium, gm	4.0	12.0	4.0	12
Phosphorus, gm	4.57	4.57	4.57	4.57
Potassium, gm	5.99	5.99	5.99	5.99

Table 5-2 A: Prevention Phase Group A (OVA)

	Prevention Phase Group A (OVA)			
	Control Diet (Control) n=5	*High Ca Diet (HC) n=5*	*Leucine Diet (Leucine) n=5*	*High Ca +Leucine Diet (HC + L) n=5*
Assigned Diet **(2 days before i.p.)**	Control	HC	Leucine	HC + L
i.p. Injections **(Day 0, 4, 7)**	OVA	OVA	OVA	OVA
Aerosol Challenges **(Day 12–47)**	OVA	OVA	OVA	OVA
Sacrifice	**Day 48**	**Day 48**	**Day 48**	**Day 48**
Sample Size	5	5	5	5

Table 5-2 B: Prevention Phase Group B (PBS)

	Prevention Phase Group B (PBS)			
	Control Diet (Control) n=5	*High Ca Diet (HC) n=5*	*Leucine Diet (Leucine) n=5*	*High Ca +Leucine Diet (HC + L) n=5*
Assigned Diet **(2 days before i.p.)**	Control	HC	Leucine	HC + L
i.p. Injections **(Day 0, 4, 7)**	PBS	PBS	PBS	PBS
Aerosol Challenges **(Day 12-47)**	PBS	PBS	PBS	PBS
Sacrifice	**Day 48**	**Day 48**	**Day 48**	**Day 48**
Sample Size	5	5	5	5

Table 5-2 C: Treatment Phase Group C (OVA)

	Treatment Phase Group C (OVA)			
	Control Diet n=5	*High Ca Diet n=5*	*Leucine Diet n=5*	*High Ca +Leucine Diet n=5*
Assigned Diet **(2 days before i.p.)**	Control	Control	Control	Control
i.p. Injections **(Day 0, 4, 7)**	OVA	OVA	OVA	OVA
Switch to Experimental Diet	Control	**Day 10** (HC)	**Day 10** (Leucine)	**Day 10** (HC+ L)
Aerosol Challenges **(Day 12-47)**	OVA	OVA	OVA	OVA
Sacrifice	**Day 48**	**Day 48**	**Day 48**	**Day 48**
Sample Size	5	5	5	5

Table 5.2 D: Treatment Phase Group D (PBS)

	Treatment Phase Group D (PBS)			
	Control Diet n=5	*High Ca Diet n=5*	*Leucine Diet n=5*	*High Ca +Leucine Diet n=5*
Assigned Diet **(2 days before i.p.)**	Control	Control	Control	Control
i.p. Injections **(Day 0, 4, 7)**	PBS	PBS	PBS	PBS
Switch to Experimental Diet	Control	**Day 10** (HC)	**Day 10** (Leucine)	**Day 10** (HC+ L)
Aerosol Challenges **(Day 12-47)**	PBS	PBS	PBS	PBS
Sacrifice	**Day 48**	**Day 48**	**Day 48**	**Day 48**
Sample Size	5	5	5	5

polypropylene cages at room temperature of 22°C ± 2°C and regime of 12 h light/dark cycle. The animals had free access to water and their experimental food throughout the study. All animals were checked daily for any signs of disease or death and moribund animals (as defined by the facility veterinarian) were humanely euthanized. Weight was measured to the nearest gram at the beginning of the experiment and then weekly until the end of the study. At the end of the study (~7 weeks) all animals were humanely euthanized by administration of sodium pentobarbital and subsequent exsanguination by severing the vena cava. Blood was immediately collected from the vena cava supply, allowed to clot at room temperature, and centrifuged to obtain serum. Immediately after euthanasia, bronchoalveolar lavage (BAL) was performed and the lungs were harvested. The left lung was fixed in 4% formalin for subsequent H&E staining and the right lung was flash frozen in liquid nitrogen for later experiments.

This study and all animal procedures were performed under the auspices of a University of Tennessee Institutional Animal Care and Use Committee-approved protocol and in accordance with PHS policy and recommendation of the Guide.

5.3.2 Induction of Allergic Airway Inflammation

The sensitization protocol was started at six weeks of age (Day 0 of the study protocol). Briefly, half the animals in both phases (prevention and treatment) (n=40 total) were sensitized with 50-µg i.p. injections of chicken egg albumin (ovalbumin [OVA]; Grad V, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 mL sterile phosphate buffered saline (PBS) on Days 0, 4, and 7 (28). Starting on Day 12, animals were challenged by aerosol nebulization delivery of ovalbumin. Animals were exposed to a solution of 2.5% OVA dissolved in PBS aerosolized by delivery of compressed air to a nebulizer for 30 min/day, 3 days/week (to allow at least a day in between each challenge)

from Day 12 to Day 47 (29). Non-sensitized or control animals for the disease model from both phases (n=40) were subjected to the exact same procedures on the same days but were not exposed to OVA. Instead PBS alone served as vehicle for the i.p. injections as well as nebulization deliveries.

5.3.3 Bronchial Alveolar Lavage Fluid (BALF) and Serum

Lungs were lavaged twice with 1 mL of cold PBS and the lavagates were pooled and placed on ice until centrifuged at 400 x g at 4°C for 10 min. Supernatant was collected and stored at -80°C for later experiments. Cell pellets were resuspended in Hank's Balanced Salt solution and an Advia 120 hematology system (Siemens Healthcare, Tarrytown, NY) was used to assess the total number of cells and estimate leukocyte distributions. Aliquots of cells were also centrifuged onto glass slides at 800 rpm for 10 min by an Aerospray 7120 cytocentrifuge (Wescor, Inc. Logan, UT). These slides were air-dried, and then stained with a methanolic Wright's stain using the AerosprayPro 7151 (Wescor, Inc. Logan, UT). Cell differentials were determined by counting 300 cells under 400X magnification by a certified pathologist in the veterinary diagnostic pathology lab at the University of Tennessee Knoxville School of Veterinary Medicine. Blood was collected from the severing of the vena cava. Serum was isolated by allowing blood to clot at room temperature and subsequent centrifugation. These protocols were adapted from those described by Williams et al (30).

5.3.4 Staining Lung Tissue Sections

Lung lobes were fixed in 4% formalin and paraffin embedded. These lung sections were stained with haematoxylin and eosin following manufacturer's recommendation (Newcomer Supply, Middleton, WI, USA). Stained lung sections were

then scored by degree of epithelial hyperplasia height to assess inflammation. The University of Tennessee-Knoxville attending veterinarian determined epithelial hyperplasia scores.

5.3.5 Epithelial Hyperplasia in the Lung (HypEpi)

Epithelial hyperplasia is characteristic of the initial stages of airway remodeling and indicates inflammation and the onset of asthmatic disease. Epithelial hyperplasia was pathologically assessed by a score of 0=absent of hyperplasia, 1=twice normal height, 2=three times normal height, 3=four times normal height. The higher the score indicates the higher degree of inflammation and airway remodeling.

5.3.6 Inducible Bronchus-Associated Lymphoid Tissue (iBALT)

Inflammation in the lung facilitates the emergence of ectopic lymphoid tissue termed inducible bronchus-associated lymphoid tissue (iBALT). This tissue can be found throughout the lung when inflammation or infections are present (31). Here we used the degree of iBALT present in the lungs of the study mice to assess lung inflammation. Bronchial associated lymphoid tissue was scored as 0=normal or no inducible lymphoid tissue present, 1= present around bronchi and larger bronchioles, 2= present around smaller bronchioles, 3= more around smaller bronchioles, by the attending veterinarian at the University of Tennessee Knoxville.

5.3.7 Statistical Analysis

Results are shown as mean (±SEM) values. Comparison of means between all diet groups was performed by one-way ANOVA. Before one-way ANOVA data was tested for equal variances (Levene's test) and normality. Differences among means were assessed by Tukey's HSD. Student's *t* test was used to test for weight gain differences

between (OVA) and (PBS) animals in both phases within diet groups. A *P* value of less than 0.05 was regarded as indicative of a significant difference. Chi-squared was used to assess categorical data (pathological degree scoring) and a value of less than 0.05 was regarded as indicative of a significant difference.

5.4 Results

There were significantly ($p<0.05$) more polymorphonuclear cell leukocytes (PMNs) including eosinophils and neutrophils present in the bronchoaveolar lavage fluid (BALF) of the OVA animals versus the PBS animals. There was also a much higher degree of inflammation in the lungs of OVA-sensitized and challenged animals as scored by inducible bronchus associated lymphoid tissue as compared to PBS vehicle animals (Table 5-3). Further, the degree of epithelial hyperplasia was increased in OVA challenged animals as compared to PBS challenged animals (Table 5-3). There were no significant differences in weight gain throughout the study or abdominal fat between diet groups in OVA or PBS treated animals. However, prevention phase animals treated with (OVA) gained significantly less weight throughout the study than (PBS) animals in all diet groups ($p<0.01$) (Figure 1). The same trend was observed in the treatment phase although the only significant difference was observed in leucine diet animals ($p<0.05$) (Figure 2). Prevention phase Group A OVA treated animals fed the combined high calcium and leucine diet had significantly fewer eosinophils present in BALF when compared to control diets ($p<0.05$) (Figure 3A). However, there no significant differences in neutrophils present in the BALF of Group A (OVA) animals between diets. Treatment phase Group C (OVA) animals showed no significant differences in the eosinophils

Table 5-3: Pathology Scores of Lung iBALT and HypEpi Across Study

	Pathology Scores				
	Prevention Phase OVA (Group A)	Prevention Phase PBS (Group B)	Treatment Phase OVA (Group C)	Treatment Phase PBS (Group D)	
Mean iBALT Score	1.0952	0	1.3182	0.0238	PBS animals had significantly less iBALT compared to OVA treated animals
Mean HypEpi Score	2.8333	0	1.5455	0.1905	PBS animals had significantly less HypEpi compared to OVA treated animals

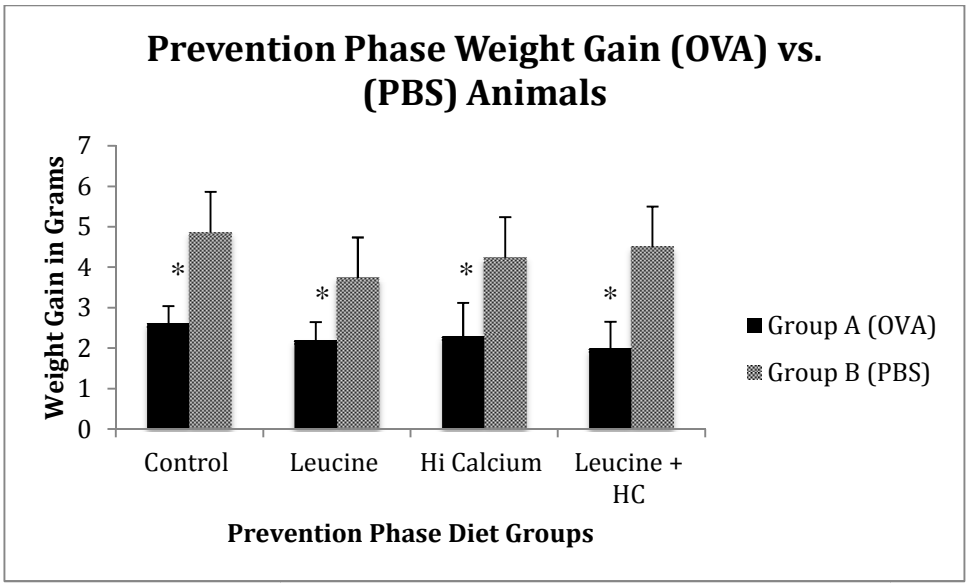

Figure 5-1: Prevention Phase Weight Gain Group A (OVA) vs. Group B (PBS) Animals. Values are expressed as mean ± SEM, (n=6). *Significantly different from same diet in opposing group, ($p < 0.05$).

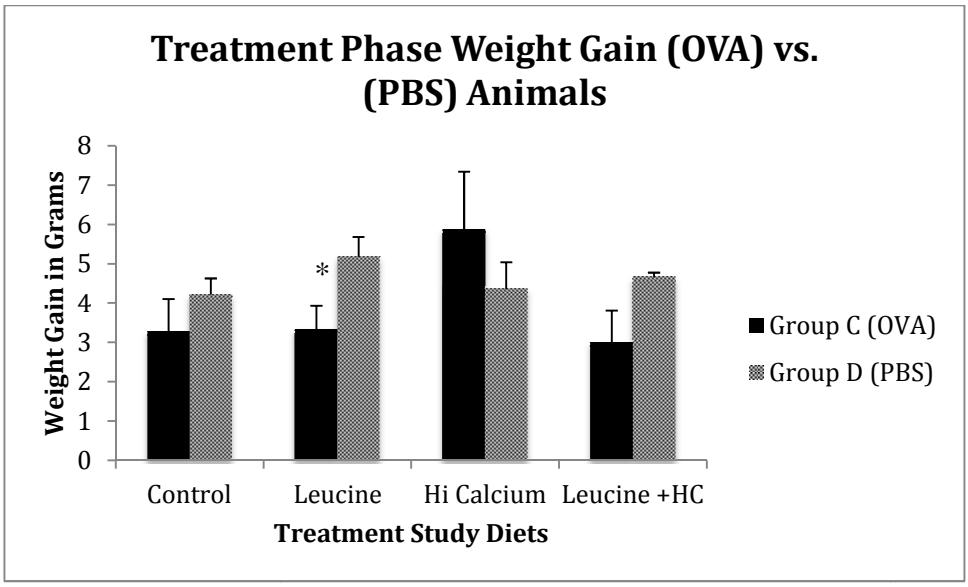

Figure 5-2: Treatment Phase Weight Gain Group C (OVA) vs Group D (PBS). Values are expressed as mean ± SEM, (n=5). *Significantly different from same diet in opposing group, ($p < 0.05$).

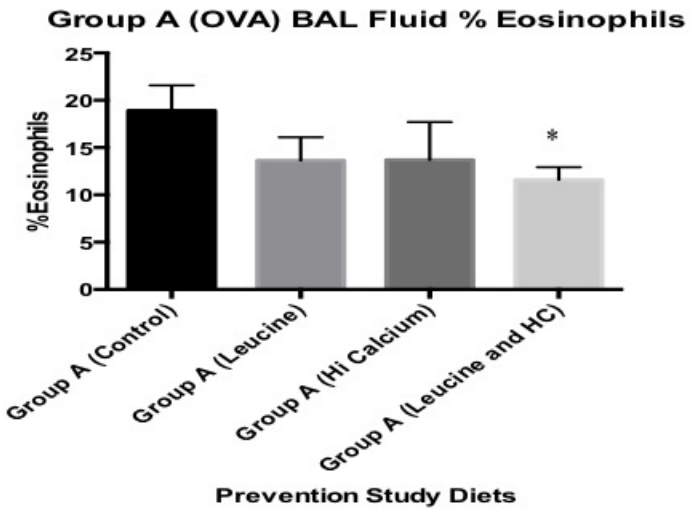

Figure 5-3 A: Group A (OVA) BALF Percentage of Eosinophils. Values are expressed as mean ± SEM, (n=6). *Significantly different from control CM, ($p < 0.05$).

present in BALF between diets (Figure 3B). Neutrophils present in BALF collected from treatment phase Group C (OVA) animals were reduced in the high calcium versus control diet, although this difference only approached significance ($p<0.07$) (Figure 4). Inducible bronchus associated lymphoid tissue (iBALT) (Figure 5) and lung epithelial hyperplasia (HypEpi) (Figure 6) and (Figure 7) as indicators of inflammation and airway remodeling were not significantly different across diets. However, the control diet animals had the largest percentage (33.3%) of iBALT scores in the severe category, while 40% of the High Calcium + Leucine diet animals were scored in the minimal to no iBALT category and none were scored in the severe category.

Serum adiponectin levels measured in prevention phase animals were not significantly different between diets (Figure 8). OVA treated animals (Group A) serum adiponectin level was significantly higher ($p<0.01$) than PBS (Group B) animals.

5.5 Discussion

Calcium and leucine supplementation of high fat diets were found to have only a modest influence on airway inflammation in a murine model of asthma. A more prominent result was expected based on previous studies where a significant reduction of inflammatory and oxidative stress was achieved in obese mice by similar interventions (21, 25). The reduction in obesity-induced inflammation observed in these studies was at least in part due to the increase of the anti-inflammatory adiponectin and decrease of pro-inflammatory TNFα secretion from adipose tissue. Studies where comparable asthma models were utilized such as Shore et al (32) showed significant improvement of disease symptoms with the exogenous administration of adiponectin. Medoff et al (28) further supported the role of adiponectin in asthmatic disease and Blacquiere et al (33) was

helpful in deciding sample size estimates for our female BALB/c model. Serum adiponectin levels were not significantly different in this study despite our previous studies where adiponectin levels were significantly changed with dietary intervention.

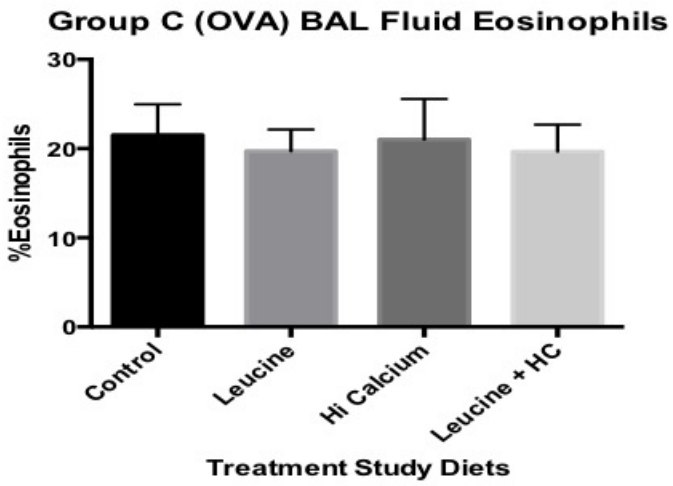

Figure 5-3 B: Group C (OVA) BALF Percentage of Eosinophils. Values are expressed as mean ± SEM, (n=5).

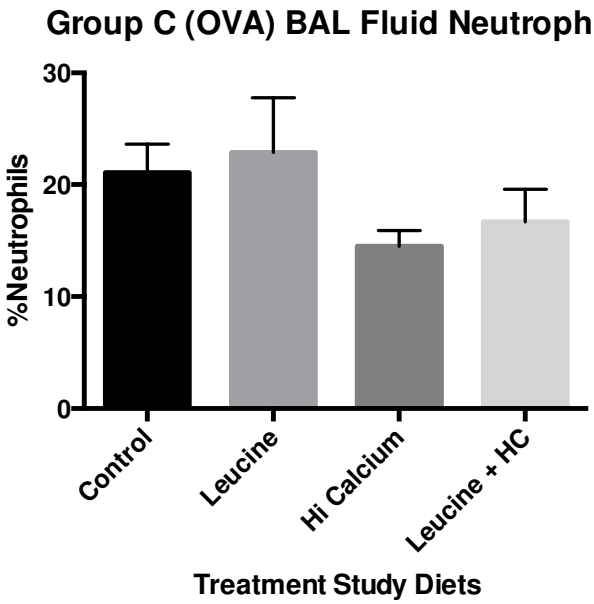

Figure 5-4:Group C (OVA) BALF Percentages of Neutrophils. Values are expressed as mean ± SEM, (n=5).

Table 5-4: Group A (OVA) Pathology Scores iBALT

	Group A (OVA) Pathology Scores iBALT				
	Control	Hi Ca	Leucine	Hi Ca + Leucine	Comment
Severe (2.0)	33.33%	0%	20%	0%	Control diet animals had the largest percentage of scores in the Severe category
Moderate (1.0 -1.5)	66.667%	100%	80%	60%	High calcium diet animals contained all their scores in the Moderate category
Minimal (0.0-0.5)	0%	0%	0%	40%	Almost half of the Hi Ca +Leucine diet animals were scored in the minimal to no iBALT category

Table 5-5: Group C (OVA) Pathology Scores iBALT

	Group C (OVA) Pathology Scores iBALT				
	Control	Leucine	Hi Ca	Hi Ca + Leucine	Comment
Severe (2.0)	33.33%	20%	60%	16.667%	High Calcium diet animals had the most scores in the Severe category
Moderate (1.5)	0%	0%	0%	33.33%	The group with highest percentage of animals with Moderate iBALT were fed the Hi Ca + L diet
Mild (1.0)	50%	60%	40%	50%	Almost half of all the animals in each diet group scored in the Mild category
Minimal (0.0-0.5)	16.6667%	20%	0%	0%	Leucine diet animals had the highest percentage in the Minimal category followed by Control diet animals

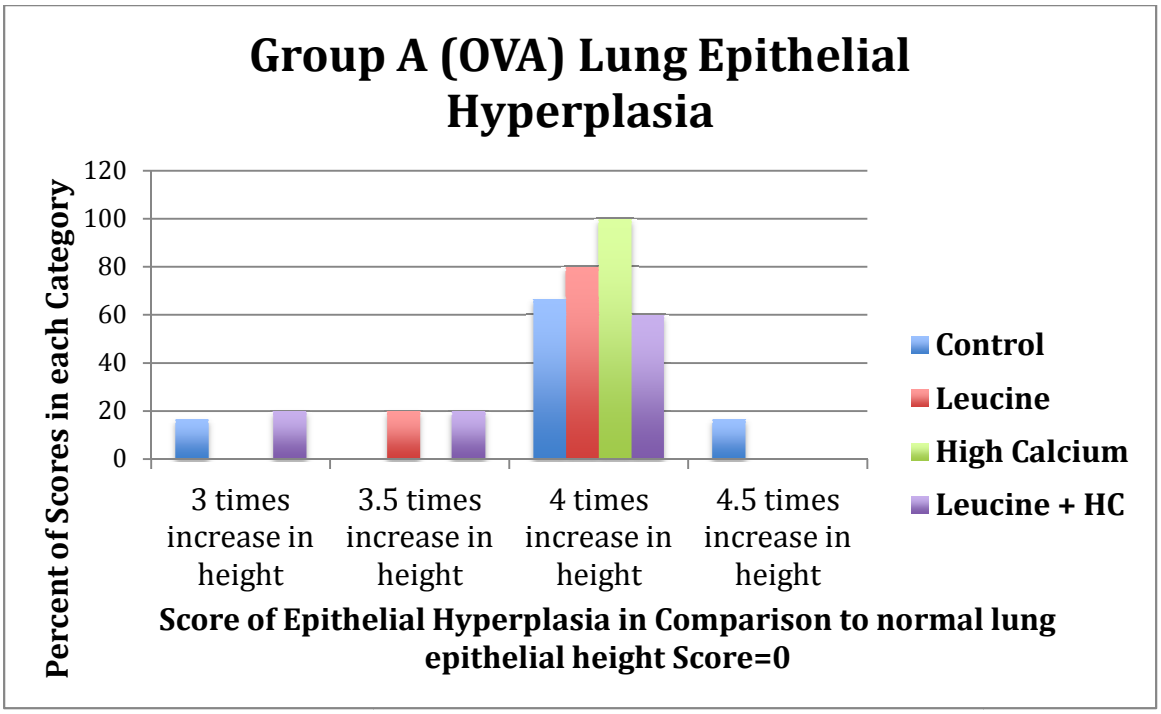

Figure 5-5: Group A (OVA) Lung Epithelial Hyperplasia (HypEpi) Pathology Scores.

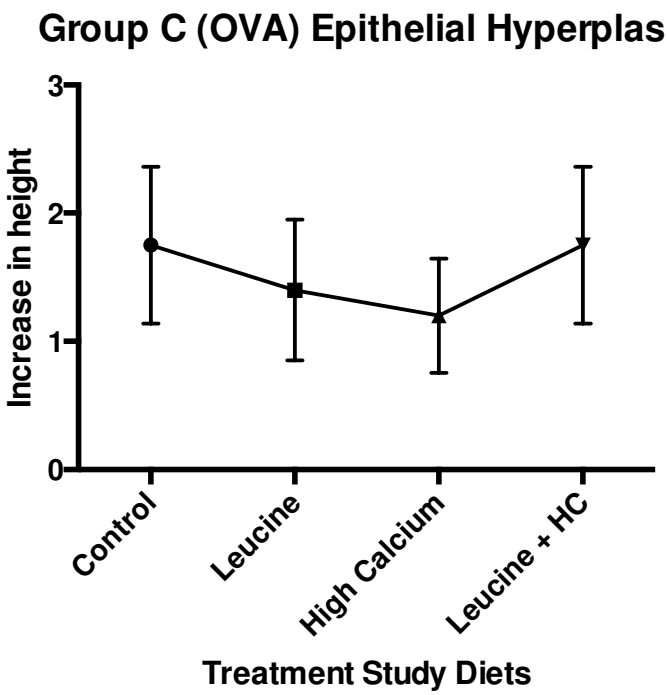

Figure 5-6: Group C (OVA) Pathology Scores HypEpi

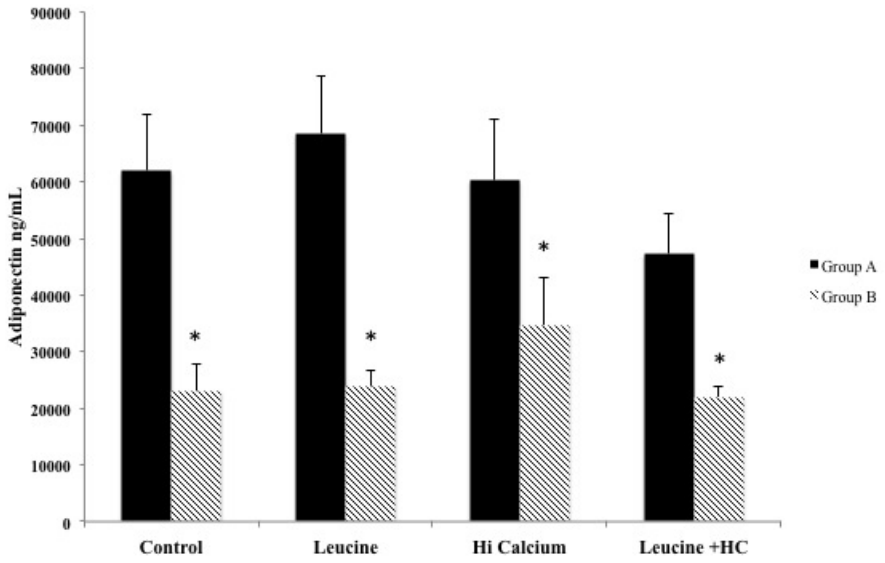

Figure 5-7: Prevention Phase Group A (OVA) and Group B (PBS) Adiponectin levels measured in collected serum. Values are expressed as mean ± SEM, (n=5). *Significantly different from animal fed same diet in opposing group, ($p < 0.05$).

However, in this study it is likely that the subtle dietary interventions used to increase endogenous adiponectin secretion may have been more pronounced in a larger sample size and longer study duration. Serum adiponectin levels in prevention phase OVA animals were significantly higher than PBS animals ($p<0.01$). The higher adiponectin levels in Group A OVA compared to Group B PBS animals reflects the observation that OVA animals also weighed significantly less than PBS animals. Treatment phase animals did not yield the same results as prevention phase animals, which suggests that experimental diet exposure time significantly impacted results. Accordingly, (BALF) neutrophils in treatment phase OVA animals fed high calcium diets appeared reduced compared to control diet animals and though not significant it was a trend not observed in prevention phase subjects. Also, in prevention phase all OVA treated animals gained significantly less weight than their PBS counterparts ($p<0.01$) but this trend was not significant in treatment phase animals with the exception of leucine diet mice ($p<0.05$).

Our previous studies were conducted in aP2-agouti transgenic mice that are genetically predisposed to develop obesity but were not an appropriate model for asthma (21). The current study was conducted in BALB/c mice; although this is an established asthma model, BALB/c mice are lean, making it difficult to use in the study of dietary modulation of airway function in obesity. By introducing high fat diets to BALB/c mice we anticipated the generation of excess adiposity to produce an asthma model incorporating characteristics of obesity. The combination of a mixed disease model, sample size/power, and duration of the study may have hindered hypothesized results. Still, high calcium and leucine-supplemented diets were able to significantly reduce the

amount of eosinophils present in BALF of prevention phase OVA animals compared to control diet mice. This decrease may have contributed to the lower pathological scores of lung inflammation (iBALT) and airway remodeling (HypEpi) found in this group compared to control diet animals although these trends were not significant. Results from this study give only a modest indication of the potential of calcium and leucine in attenuating obesity associated airway inflammation further research in this area is still warranted.

5.6 Strengths, Weaknesses, and Limitations

The strength of this study is the generation a murine asthma model with lower airway inflammation. Additionally, the BALB/c mouse strain is the ideal model for translational asthma studies. However, the model is also a limitation as a lean mice strain it is difficult to use in dietary modulation of airway function of obesity studies. For this reason the BALB/c mouse model is both a strength (asthma) and a limitation (obesity) of this study. The duration of the study and sample size is also a weakness of the study

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Part Six

Summary and Conclusions

Leucine and calcitriol appear to have opposing effects on adipocyte pro-inflammatory cytokine secretion. Experiments conducted *in vitro* suggest that leucine provided an anti-inflammatory effect by decreasing leukocyte activation markers (CD11b), decreasing adhesion molecule expression (ICAM-1), and subsequently decreasing leukocyte adhesion to smooth muscle cells and endothelial cells. Extension of these findings to an established murine model of asthma did result in a significant decrease in eosinophils present in the bronchoalveolar lavage fluid (BALF) of BALB/c mice fed a high calcium and leucine supplemented high fat diet compared to control diet animals. Overall, however the animal study showed only modest effects of calcium and leucine supplementation. The BALB/c mice strain (although ideal for asthma studies) is lean, making high-fat diet induced obesity difficult and perhaps compromising the desired model for this study. The prominent eosinophilic rich inflammation assessed by collected BALF fluid as opposed neutrophilic inflammation suggests the obesity associated asthmatic phenotype was not achieved in the BALB/c model. Nonetheless, calcium and leucine supplementation did decrease eosinophil infiltration likely a result decreased of leukocyte CD11b and lung endothelial cell ICAM-1 expression observed in the *in vitro* studies.

Vita

Patricia Louise Brown was born on January 18th, 1985 to her parents David E. and Judith Brown in Crossville, Tennessee. Graduating high school early to start on her undergraduate degree Patricia attended Roane State community college before achieving the top ten percent of her graduating class from Cumberland County High School. In the Fall of 2003, Patricia began her undergraduate curriculum at the University of Tennessee Knoxville and graduated with a bachelor's degree in Biology with a concentration in Microbiology from the college of Arts and Sciences in 2008. After receiving her B.S. degree Patricia began to conduct research in the biochemistry, cellular and molecular biology (BCMB) department of the University of Tennessee Knoxville under the supervision of Dr. Rose Goodchild. In 2009, Patricia joined the Center of Environmental Biotechnology of UTK as a Research Specialist II working under the guidance of Dr. John Biggerstaff. Patricia started graduate school in the Nutrition Department of UTK in the fall of 2010 and completed her doctor of philosophy (PhD) degree in Nutritional Sciences with a concentration in Cellular and Molecular Nutrition and cognate in Comparative and Experimental Medicine under the principal investigator Dr. Michael Zemel graduating in August 2014.